

Rethinking on weapons clean-up

Science can make a critical contribution to the massive US effort to clean up sites once devoted to manufacturing nuclear armaments — but only if the Department of Energy is effectively reformed.

THE clean-up of the nuclear weapons production complex in the United States presents a daunting array of technical challenges, which science will play an important role in meeting. Hazel O'Leary's energy department has made some progress toward injecting technical and administrative cohesion into the vast programme. If, as expected, O'Leary leaves at the end of the year, the acceleration of this process will be the top priority of her successor.

The litany of problems surrounding the \$6-billion-a-year programme began at its outset, in 1989, with the failure properly to integrate science and technology into its core. As a result, the programme has lacked technical direction. Tens of thousands of people are engaged in the work, which is supposed to lead to the environmental remediation of a chain of huge former weapons production plants across the United States. But experts have fiercely criticized the Department of Energy (DoE)'s management of the programme, claiming that many of its approaches are expensive and technically misguided (see pages 375–379).

So what must be done to reform it? The department — at the insistence of the Congress — has taken one step in the right direction this year, establishing a \$50-million-a-year programme of basic research directed at the clean-up technologies of the future. The Environmental Management Science Program will fund chemists, biologists and others to pursue more efficient ways of disposing of high-level waste, and various biological and chemical means of erecting underground 'barriers' to block the dispersal of toxins and radionuclides.

The first round of grants has been split evenly between scientists at universities and at the DoE's own laboratories. The typical grant for the latter is \$1 million — three times the size of the typical university grant, reflecting the high overheads associated with work in the laboratories. If the department is to get value for the taxpayers' money, it must either reduce overhead costs at its laboratories, or direct more of the money at the universities.

The time is also ripe for an extensive revision of the objectives of the programme, based on a scientific assessment of the risks posed by different hazards on the sites. When the clean-up problem was first exposed to the public, its natural response was to demand simultaneous remediation action on all fronts. It is becoming clear that this approach is technically and fiscally unsustainable: the problems must be prioritized on the basis of risk.

Such revisions will have to be agreed with local state governments and the Environmental Protection Agency. In the past, an energy department in search of a quiet life has reached agreements with those parties which it is now unable to implement. In renegotiating the agreements, the department must be much firmer than it has been, resisting the temptation to promise actions that its own scientists and engineers do not support.

O'Leary's strongest legacy has been her 'openness initiatives', which have helped to make the public (and local environmental activists, in particular) more inclined to believe the department. Her successor must take advantage of this legacy to win consent for patient and technically sound approaches to clean-up. The introduction of performance-based contracts at key sites such as Hanford provides additional grounds for optimism.

But the department remains vastly bureaucratic, with large and powerful local offices vying with its headquarters in Washington,

DC, for control of the programme. And many in Congress continue, perversely, to measure the success of the programme by the number of jobs it retains on the sites. In the resultant dog-fight for resources, the investments that the programme most needs tend to suffer. John Myers (Republican, Indiana), chair of the energy appropriations subcommittee in the House of Representatives, is to be congratulated on breaking this cycle to make funding available for the new science programme.

Doubts remain, however, about the ability of the Department of Energy as now constituted to reform the clean-up programme. Last year, a report by a panel chaired by the industrialist Bob Galvin was highly critical, calling for a research programme worth \$200 million initially, rising to \$400 million a year, to find new approaches to clean-up. Galvin also recommended the promotion of the assistant secretary for environmental management to the rank of under secretary.

Neither recommendation has been implemented. Instead, the former assistant secretary, Tom Grumbly, after coming closer than any of his predecessors to getting to grips with the problems, was promoted this summer. A new assistant secretary, Alvin Alm, has arrived, with his own vision of maximizing clean-up over the next ten years. Laudable as this goal may be, it highlights the impression of stop-go management which has been the bane not just of the clean-up programme but of the entire department.

Republicans in Congress continue to call for the abolition of the DoE. They have already scaled back its energy supply mission and say the weapons programme should go to the Department of Defense and the laboratories to the National Science Foundation. But the abolitionists never mention clean-up, which is as expensive as, and more problematic than, the other three missions put together. Next month, the Republicans are defending six marginal congressional districts downriver from the former plutonium production facility at Hanford. Voters there should be told now if the party is really intent on handing over the worst environmental site in the country to the secretive hands of the Department of Defense.

Senator Pete Domenici (Republican, New Mexico), the department's best Republican friend, has floated the concept of a change in the department's status as the best means of strengthening its component parts. If DoE became an agency, like the National Aeronautical and Space Administration (NASA), the argument goes, it could find direction under a strong and politically independent administrator. This approach has much to commend it.

In a wholly rational world, the clean-up programme might cost about one-tenth of its current budget — the irrationalities stemming from politicians' need to preserve constituents' jobs, excessive bureaucracy and the wish of US citizens to see the problems tackled rather than mothballed. But with appropriate reforms and prioritization, money can be redeployed to boost research, as Galvin rightly recommended. Early in the next century, technology for clean-up will drastically improve, and public demand for it will recede, subject to accurate assessment of the risks posed by residual radioactive waste. At some point, expectations and capabilities are likely to coincide. Science has a pivotal role to play in helping the government to reach that point as quickly and as cheaply as possible. □



Closure of astrophysics laboratory blamed on Enola Gay 'fallout'

Washington. The US National Air and Space Museum in Washington, DC, is to close its Laboratory of Astrophysics, angering astrophysicists who point to the laboratory's strong track record in research and teaching.

The closure of the laboratory is being widely viewed as retribution against Martin Harwit, an astrophysicist and former director of the museum, who founded the small laboratory in 1987. Harwit was forced to resign last year after veterans' organizations and congressmen objected to plans for an exhibition about Enola Gay, the aircraft that dropped the first atomic bomb (see *Nature* 375, 95; 1995).

The Smithsonian board of regents asked for a review of the laboratory last year, after critics had asserted — falsely — that its main function had been to support Harwit's own research. An external panel, chaired by Maxine Singer, head of the Carnegie Institution, rebutted criticism of the laboratory. But Don Engen, the museum's new director, told staff last month that the laboratory will close by the end of this year.

With a core staff of only three, whose salaries have been paid by the Smithsonian, the laboratory had attracted \$4 million in external research grants since its inception. Its researchers have published 150 papers,

conducting top quality research in a range of special topics, including optics for high-transmission infrared spacecraft systems, and spectroscopy for velocity-resolved studies of planetary atmospheres.

Perhaps the laboratory's most important function, however, has been to provide the museum with scientific advice on its exhibits, and to help with its external education efforts, including the widely circulated IMAX movies *Cosmic Voyage* and *Destiny in Space*.

Howard Smith, the head of the laboratory, declines to comment on the circumstances surrounding the planned closure. But colleagues of staff members point out that the closure will not save the Smithsonian any money, as external grants cover its operating costs. "This has to do with the Enola Gay exhibition and Martin Harwit's resignation, and [the Smithsonian] wanting to make a clean sweep of things that Harwit created," one said.

Engen was unavailable for comment. But his deputy, Don Lopez, says the laboratory is closing because it is "not right in our primary core function" of putting on displays, which Congress told the museum to concentrate on. Lopez says that the museum has already reduced its staff from 235 to 213 following budget cuts, and has been instructed to reduce the number to 201 by

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Seeing red: staff use a laser to develop optics for infrared spacecraft systems.

the end of next year.

Lopez denies that the laboratory is being closed because it was Harwit's creation: "Harwit is already gone — how could it be retribution against him?" The museum will retain its other, larger laboratory, the Center for Earth and Planetary Studies, whose staff are mainly geologists. Lopez says that laboratory is being retained because it "provides a unique function" and has existed "ever since the museum was founded" in 1976.

Harwit says that the closure of the astrophysics laboratory is "a step backwards" that could have been avoided within the staff reductions that the museum is making. "I set up the laboratory because I felt that an essential part of telling the story of space required people who were actively involved in space science," he says. The museum's exhibits and education programmes "need that kind of expertise. You can't do that if you just have people who read about it in the *Encyclopaedia Britannica*".

A proposal to integrate some of the laboratory's activities into the Smithsonian Astrophysical Laboratory in Cambridge, Massachusetts, directed by Irwin Shapiro, was rejected last week by the Smithsonian management. Lopez says that the museum will be able to call on the Cambridge laboratory for scientific advice as required. Two of the astrophysics laboratory's three permanent staff have now found jobs elsewhere.

Colin Macilwain

Governor blocks genetic property right

Washington. The Republican governor of New Jersey — home to dozens of biotechnology companies, and more major US pharmaceutical companies than any other state — has vetoed a bill creating a property right to genetic information, warning that it could "chill" research in the state.

The Genetic Privacy Bill had earlier been unanimously approved by the state legislature. Governor Christine Todd Whitman issued a conditional veto of the bill last month, arguing that its creation of a property right was "not needed" to protect privacy.

Those pushing for the veto included the Biotechnology Industry Organization, whose president, Carl Feldbaum, called the bill "so badly written" that it would have eliminated much basic research, including many clinical trials.

The 'conditional' veto means that, if the changes suggested by Whitman are

incorporated, the bill can be passed into law by a simple majority, rather than the two-thirds normally needed to override a veto. Whitman has asked for the property right provision to be dropped, and those primarily responsible for drafting the bill say they will remove it.

"The most important issue is to protect New Jersey residents from genetic discrimination. Therefore it's most appropriate to move the bill forward," says Steven Andreassen, a legislative aide to Jack Sinagra (Republican), a key Senate backer of the original bill. But he says that Sinagra still opposes in principle the removal of the property rights provision.

The bill was approved by the New Jersey Senate by 38 votes to nil in June, and passed unanimously by the Assembly the same month. The pharmaceutical industry is the state's largest high technology employer.

Meredith Wadman

ESA faces economies to pay for Ariane launch failure

Paris. The explosion of Europe's Ariane-5 satellite launcher on its maiden flight last June will add ECU288 million (US\$362 million) to the cost of the programme, and delay the full commercial exploitation of the launcher by a year to early 1998.

These and other details of how the Ariane-5 programme is to be resumed following the explosion were announced last week at a press conference in Paris by Jean-Marie Luton, director general of the European Space Agency (ESA), and Alain Bensoussan, president of the French space agency (CNES).

Two further qualification flights of Ariane-5 are now planned, the first next April and the second next September. The first will carry only a payload of instruments to evaluate the performance of the launcher, and a 500-kg AMSAT satellite of the International Association of Radio Amateurs. The second will carry a demonstration model of a planned European atmospheric re-entry vehicle.

These dates are only targets, however, and may be postponed if necessary. Indeed, before the next launch, ESA intends to go beyond the recommendations of the explosion inquiry board and to re-examine the entire qualification system.

The Ariane-5 launch failed because of software errors that went undetected during tests and caused the rocket to veer off course and break up. The re-examination will consider in particular how the launcher would perform in highly 'degraded modes' — after the failure of one or more systems and their back-ups — and take steps to cover such eventualities.

The cost of the work required is estimated at ECU154 million, while the extra qualification flight will cost ECU134 million, giving a total bill of ECU 288 million. This will make a large hole in the ECU1.72 billion budget agreed for the programme between 1996 and 2003 (see *Nature* 377, 667; 1995).

Luton says that the money will have to be found from within existing budgets. Economies will be needed in other ESA programmes, such as plans to expand Ariane-5's capabilities, says Luton. He is also hoping to obtain contributions from industrial groups involved in the programme.

ESA's space science programme is unlikely to be directly affected by the economies. Its budget is agreed every three years by ESA ministers, and any reduction would require a unanimous vote. This would "be opposed", says Roger

Bonnet, head of the science programme.

But Bonnet warns that money could be taken from the space science programme in more indirect ways, such as charging it for services that have until now been provided free. Similarly, the increase in pressure on already strained budgets may make it more difficult for member states to afford the costs of building their payloads and will increase pressure for the space science programme to contribute to these costs.

Space science was notably absent from the agenda at last week's press conference, and no mention was made of plans to replace the Cluster project (see *Nature* 383, 111; 1996), whose four satellites were lost in the explosion. Ominously perhaps, ESA's ECU288-million estimate of the financial

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Up in smoke: the first flight of Ariane-5 in June ended when it veered off course shortly after launch.

consequences of the explosion also includes no reference to the costs of replacing Cluster, which was intended to study the electrical and magnetic field environment of the Earth.

Bonnet describes the omission of Cluster from last week's discussion as "a bit scandalous". He is determined to make a detailed case for a replacement mission at a meeting of ESA's science policy committee next month, and wants to reduce the "prohibitive" ECU350-million projected cost of the mission by "more than a factor of two".

The major hurdle is that the space science programme cannot afford the ECU130 million needed to launch a replacement mission on Ariane-5, and member states are proving reluctant to make the extra commitment that would be required (see *Nature* 383, 111; 1996).

While Bonnet is trying to seek a compromise with ESA's launcher division, he is also considering reducing the size of the satellites so that they could be launched on an Ariane-4. The chances of rebuilding and relaunching Cluster are "fifty-fifty", he says.

Declan Butler

Bioethics group finds 'no objection' to human gene patents

Munich. A group of ethics advisers to the European Commission in Brussels says that it has found no ethical reason why a human gene should not be patented by its 'discoverer' if it can be shown that it has a function with a specific industrial application.

But, in a statement last week, the group added that it should not be possible to patent the simple knowledge of the structure of a complete or partial gene sequence without identifying its function. Such a patent would not meet the primary requirements of novelty, inventive step and industrial application.

The group was asked by the commission last April to consider the ethical implications of patenting material of human origin, as part of a wide consultation process intended to help smooth the passage of a European directive on the patenting of biotechnological inventions. An earlier directive failed to win the vote of the European Parliament last year, partly because of ethical concerns (see *Nature* 374, 103; 1995).

The group is made up of nine bioethics experts, and is chaired by Noëlle Lenoir, a member of France's constitutional court. In a formal opinion delivered to the commission, the group says that, in accordance with the ethical principle that the human body should not be commercialized, a person donating samples from which a patented gene is derived is not entitled to payment. But the person should be fully informed and provide full consent. Patents should not be granted on a human body or parts of a human body, says the group.

One member of the group, Dieter Mieth, professor of scientific ethics at Tübingen University in Germany, dissented, saying that human genes should be patentable only if their structure has been altered.

The advisory group's opinion has been welcomed by the commission, as it coincides with the thrust of the new directive, which faces its first reading in the European Parliament early next year. According to Willi Rothley, deputy chairman of the parliament's committee of legal affairs, which will introduce the directive, a "clear majority" of Members of the European Parliament are now in favour of it. This is a result of the commission's extensive consultation process and consequent modification of the text (see *Nature* 378, 756; 1995).

Approval of the directive by the Council of Ministers, which requires a simple majority vote, also seems certain, with only Denmark likely to voice objection.

Alison Abbott

Bias alleged in Japanese university awards

Tokyo. A new programme that injects much needed support into research in Japan's universities, but uses a hierarchy of committees rather than conventional peer review to select project leaders, appears to have favoured institutions and individuals represented on the programme's selection committees, in particular Tokyo University.

The programme, 'Research for the Future', was launched this year by the Japan Society for the Promotion of Science (JSPS) with an initial budget of ¥11 billion (US\$100 million) from the Ministry of Education, Science, Sports and Culture (Monbusho). It will support research projects for five years with an average annual budget of about ¥100 million (US\$1 million) each.

The awards are particularly attractive because they are much more flexible than the traditional grants awarded by the ministry and can, for example, be used to employ postdoctoral fellows. Furthermore, in a first for science in Japan, as the programme is funded as 'capital investment', it is free from the ceilings that normally restrict growth in science budgets, and is already expected to double in size next year (see *Nature* 383, 206; 1996).

But, unlike the ministry's existing grant schemes, the project grants are not awarded through open, peer-reviewed competition. Rather, project leaders are chosen in a 'top-down' fashion using a hierarchy of committees. Under this system, a top programme committee of 14 members, headed by Hiroyuki Yoshikawa, president of Tokyo University, and comprised of university presidents, directors of Monbusho institutes, and two industrialists set up three subcommittees in the fields of science and engineering, life science, and interdisciplinary research respectively.

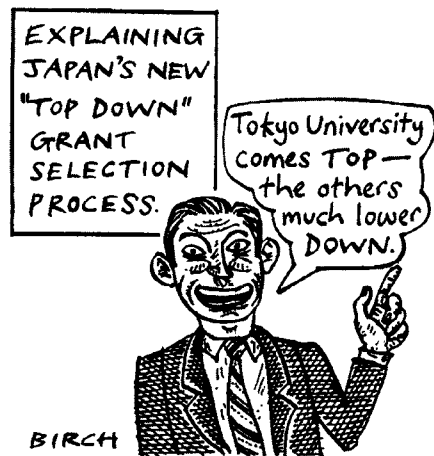
These in turn selected 17 specialist research areas for funding on the basis of suggestions from university researchers throughout Japan, and chose the members of the selection committees for each area. The selection committees then decided who the leaders of specific research projects should be. There was no open call for university researchers to make detailed grant proposals, and no peer review.

The first 117 projects selected this summer appear to show clear signs of bias in favour of selection committee members and their institutions. In particular, Tokyo University, which was by far the most strongly represented university on the selection committees, has more than twice as many leaders of projects as any other university. Furthermore, 34 out of 98 of the selection committee members were themselves chosen to head projects that received funding.

The results are "completely outrageous", says one professor at Kyoto University who has seen the complete list of project leaders

and selection committee members. He says, for example, that in genome research, four out of five project leaders are either members of the selection committee, or junior staff on the faculty of committee members.

The bias is particularly strong in science and engineering, where Tokyo University has 10 out of 41 project leaders, or 25 per cent of the awards (compared with 19 per cent for the programme as a whole, already



a relatively high figure). Furthermore, in this field an unusually large number of members of the selection committees — 15 — were chosen as project leaders.

According to a spokesman for JSPS, Yoshikawa played a key role in selecting the members of the science and engineering subcommittee, while Hiroo Imura, president of Kyoto University, was in charge of life sciences, and Leo Esaki, president of Tsukuba University, handled interdisciplinary research. According to one member of the top programme committee, these subcommittees were "critical" in determining the selection of research areas

and selection committee members.

A quarter of such committee members in science and engineering came from Tokyo University — more than three times as many as any other national university — and there were also several former professors of Tokyo University on the committees who now work at private universities but maintain strong links with Japan's leading national university.

Yoshikawa denies influencing the decisions of the selection committees. "We made a strong request that the selection committees should select the best research groups, regardless of geographical location or university," he says. But, he adds, despite a rule that committees should not select their own members, "unfortunately some — one or two — could not follow this rule".

Yoshikawa says that the new 'top-down' approach of grant selection is intended to complement the traditional peer review system, which still covers 90 per cent of the university research grants awarded by the ministry of education.

He says that in the peer review system in Japan, selection committees have no responsibility for their decisions, and that he wants to introduce more responsibility and accountability. Yoshikawa says that a committee will be set up not only to review the progress of the research projects, but also to assess the selection committees themselves. If any committees are found to be "failing" in their selection of project leaders, he adds, "they will be changed".

In defence of this year's selections, Yoshikawa points out that the selection process was extremely rushed because a law allowing the new programme to be set up was not passed by the Diet, Japan's parliament, until the end of May.

David Swinbanks

French scientists rally against racist claim

Paris. More than 600 French scientists and historians have signed a statement censuring Jean-Marie Le Pen, the leader of the extreme right-wing party the National Front, for his statement of belief in the "inequality of races". Le Pen said: "It is obvious, the whole of history demonstrates it. They don't have the same capacity for evolution."

Le Pen, who obtained 15 per cent of the votes in last year's presidential election, has also made much of the fact that blacks won more medals than whites at the recent Olympic games.

The statement drafted by Albert Jacquard, a retired geneticist, contests Le Pen's claims "in the name of scientific rigour". The text says that although all humans (except identical twins) have

different genomes, this is "in no way synonymous with hierarchy".

Populations of *Homo sapiens* have not been isolated for sufficiently long to have given rise to distinct races, says the text. But a columnist for the newspaper *Le Monde* warns that scientists may be playing into Le Pen's hands. "Arguing about the diversity of races, in the name of scientific rigour, to prove the equality of men, is to admit that this equality can be a subject of discussion," the columnist writes.

Signatories include the leading genome scientists Jean Weissenbach and Charles Auffray, the geneticist Axel Kahn, and the eminent biologists Pierre Chambon and Jean-Pierre Changeux.

Declan Butler

Loans may keep CERN collider on target

Munich. The European Laboratory for Particle Physics (CERN) in Geneva, Switzerland, may have to take out bank loans to keep on target the construction of its accelerator, the Large Hadron Collider (LHC).

Such a move would reverse a policy agreed only two years ago that all construction work should be paid for out of the annual subscriptions of CERN's 19 member states. It is a direct result of Germany's unexpected decision in July to reduce significantly its contributions for the next few years (see *Nature* 382, 285; 1996).

The severity of Germany's proposed reductions — 8.5 per cent of its previously agreed contribution for 1997 and 1998, and 9.3 per cent for the following two years — will have a major impact on the construction of the SFr2.6-billion (US\$2.08-billion) LHC, the world's largest basic research project.

CERN's committee of council, which met in Geneva last week, acknowledged that raising loans, to be paid back over a longer period of years, may be the only way to meet the goal of completing the LHC by the year 2005. The committee, which is made up of representatives of member states, rejected requests from Germany to be considered a special case.

The committee said that if Germany reduces its subscription, then all other member states will follow suit. Indeed, many countries would view the chance to save money on international projects with some relief. France, for example, has already announced plans to reduce its subscription to the laboratory next year by 2 per cent (see *Nature* 383, 289; 1996).

Contributions are decided by a formula based primarily on the size of a country's gross national product. Such reductions could undermine CERN's attempts to raise funding for the LHC from other countries,

in particular Japan and the United States, to bring forward the final completion date for the collider.

The proposed funding for the LHC had already been severely stretched before member states finally approved the project in 1994. A decision was made then to build the LHC in two phases (see *Nature* 372, 713; 1994) in order to spread construction costs over a longer period, although CERN scientists have always viewed this as a compromise that will not provide optimum scientific return.

It had been hoped that the original timetable, involving full operation in 2005, could be restored by contributions from non-member states. By last July this hope appeared justified. The United States, Japan, Russia and Canada were talking seriously about contributions that could add up to a total of SFr450 million.

But if all member states reduce their contributions in line with Germany, then these additional contributions could fail to materialize, says Maurice Jacob, who manages CERN's relations with its member states. Negotiations have been carried out on the assumption that the 2005 target date would be achieved, he says, but with such a large drop in CERN's own budget this now seems in doubt.

Member states are acutely aware of what is at stake. One member of the CERN committee said that it was "ironic" that, in its attempts to meet the criteria for joining a single European currency, "Germany is threatening the largest European cooperative project in science".

The committee expressed little sympathy for Germany's situation. In a statement issued after the meeting, Christopher Llewellyn Smith, CERN's director general, pointed out that many countries face

Luciano Maiani (right), president of Italy's particular physics research organization, the National Institute for Nuclear Physics (INFN), was last week elected president of CERN by representatives of its governing council, meeting in Geneva. Maiani, who is a professor of theoretical physics at Rome's La Sapienza university, will take over from Hubert Curien, the former French research minister, from next January. □



similar economic difficulties.

Germany's argument that the cost of reunification makes its position worse than that of other member states has made little impact, as Germany has already been given a special dispensation to compensate for this. A three-year agreement allowing Germany to reduce its contributions from 25 per cent of the total CERN budget — the ceiling for any contribution from a single member — to 22.5 per cent has been extended by two years and now ends in 1998. Nonetheless Germany remains the highest contributor to CERN.

Officials at the laboratory are preparing options that will be put to the committee in an extraordinary meeting on 7 November, before a meeting of the full council in December.

During this period, efforts will be made to persuade Germany to modify its position, although a spokesman for the German research ministry says that a change of heart is unlikely. But officials will develop a range of financial options that could be introduced to help keep the project on track if a compromise proves impossible.

Some help has come from the fact that inflation has been lower than expected in Switzerland, allowing CERN to save SFr60 million over the past two years. Llewellyn Smith has said that this can be used to help bridge the shortfall, despite the risk that CERN would be unable to cushion any future inflation that was higher than expected.

But the committee of council accepted that the only way to meet the remaining shortfall would be to take out loans and pay for the machine over a longer period. CERN did this previously to pay for its Large Electron-Proton (LEP) collider. But in 1994 CERN's council voted against loans, because of opposition from the finance ministers of many member states to the possible extra costs involved. This decision now seems likely to be reversed.

Alison Abbott

US review to examine university research

Washington. President Bill Clinton last week ordered a comprehensive review of the way federal government policies affect scientific research in US universities. The review will be carried out by the President's Council of Advisers on Science and Technology (PCAST), which has been told to report by the end of next June.

The PCAST Committee on Fundamental Science — chaired jointly by Harold Varmus, director of the National Institutes of Health, and Neil Lane, director of the National Science Foundation, together with Ernest Moniz, of the White House's Office of Science and Technology Policy — will set up an inter-agency task force to conduct the review.

According to the White House, this will focus on "what stresses [research] universities may be faced with and whether

the federal government might be contributing to them".

Judith Rodin, president of the University of Pennsylvania and a member of PCAST, says that the review will study inconsistencies in policies between government agencies which make compliance "weighty and confusing and difficult" for research institutions.

According to Lane, the review is needed to dispel various myths. "There is not a clear dialogue going on between government and higher education," he says. Priorities will include federal support for undergraduate, graduate and postdoctoral science education; the impact of Washington's policies on government-university research partnerships; and streamlining regulations without damaging researcher accountability.

Meredith Wadman

Popularity puts growing strain on Brussels

Freiberg. Research administrators were told last week that the European Commission needs the backing of the academic community if its attempts to sharpen the focus of projects funded under its next five-year Framework research programme are to succeed in the face of political challenges.

The warning came from Graham Stroud, head of the commission's research management department, in an address to a meeting organized by KOWI, the German organization of European Union liaison officers, held in Freiburg, east Germany. The commission is proposing that the number of research lines be significantly reduced in the next Framework programme, due to start in 1998, partly to increase the currently low success rate of applications.

But Stroud warned that the European Parliament and Council of Ministers, both of which must approve the programme, could demand an increase in the number of research lines to promote national interests. Researchers could help by lobbying politicians in their own countries, to impress on them the importance of not trying to spread research money too thinly, he said.

The research directorate of the commission has become a victim of its own success. According to its 1996 annual report published last week, 24,000 proposals involving more than 100,000 participants were received last year. This represents nearly a tripling of workload in the past four years, during which time the number of staff at the

commission has increased by only around 20 per cent.

This places a strain on the evaluation process, said Stroud, and has also caused the success rate of applications to plummet. It now averages 20 per cent, and in some programmes is as low as 6 per cent. The commission wants to raise the average success rate to one in three, a figure agreed with representatives of the research community during a seminar on programme management this summer.

The commission is already taking steps to try to control the situation, Stroud told the meeting. It plans to reduce the average time for approving applications from five to four months, in line with the speed of the most efficient national research councils in Europe. It is also extending to more programmes a newly introduced system for weeding out ineligible applications in advance.

But in the end "all this will only be fiddling at the edges", he said. "Everyone knows that the only way to get things under control is to reduce the number of programmes and to focus them." Member states agree with this in principle. Restricting the number of programmes heads the list of most countries' position papers on the fifth Framework programme, which takes over when the fourth programme finishes at the end of 1998 (see *Nature* 381, 634; 1996).

Yet commission officials remember the fate of a proposal that there should be only

six research programmes in the fourth Framework programme, which was put forward by the former research commissioner, Filippo Pandolfi. The number had risen to 15 by the time all member states felt that they were benefiting individually, and gave the programme their approval.

The meeting in Freiburg also learned that the future remains unclear for the task forces set up last year to coordinate the research efforts of the European Commission in particular technological areas. Jean-Marie Martin, director of the commission's Environment Institute, based at Ispra in northern Italy, who heads the task force on water, said that the task forces still had no clear idea about how they were likely to fit into the decision-making process of the commission. And he said there was still no word on how they were to be financed.

The research commissioner, Edith Cresson, suggested a year ago that the task forces' first round of work should be supported by 'top-up funds' held in reserve for the fourth Framework programme. However, because of the European beef crisis, the amount of money likely to be available has fallen from ECU700 million (US\$881 million) to around ECU200 million (US\$252 million). The Council of Ministers has yet to vote on whether all or none of this potential fund should be approved. A decision is expected before the end of the year. But it is likely to come too late for the 1997 budget. **Alison Abbott**

UK medical chief suggests 'safe' does not mean 'no risk'

London. The chief medical officer of the UK's Department of Health — currently embroiled in political controversy over whether British beef is safe to eat — has suggested standardizing the way in which risks are described to the public on a scale based on their severity.

Kenneth Calman proposes in his annual report, published last week, that the word 'safe' should, if used, "be seen to mean negligible" — itself defined as "an adverse event occurring at a frequency below one per million". It should not, he adds, "imply no, or zero, risk".

Such a definition of negligible risk would, for example, include the chances of dying from being hit by lightning, or from the radiation released by a nuclear power station. It would also, on the basis of the most recent scientific evidence, include the chances of contracting Creutzfeldt-Jakob disease (CJD) by eating meat infected with bovine spongiform encephalopathy (BSE).

Calman's suggestions are put forward in a section of his report on "topics of particular importance". They are, he says, an attempt to "present a vocabulary for debate",

adding that the perception of risk by the individual is "the most interesting but most difficult aspect of understanding risk".

He proposes dividing events into six categories, based on the chances of each occurring to an individual within any one year. These start with events of "high risk", where the risk estimate is considered to be greater than one in 100, and descend in a series of steps through "moderate", "low", "very low", and "minimal" to "negligible" risk, each encompassing events whose likelihood is an order of magnitude smaller than those in the previous category.

Calman acknowledges that any classification of a particular risk needs to be qualified by other words that may be equally important. These include whether the risk is avoidable or unavoidable;

whether it is justifiable; whether it is acceptable or unacceptable (the latter meaning that it "would not be tolerated except for special reasons — such as the use of unproven medical treatment as a therapy of last resort"); and whether the risk is "serious".

His categorization of risk has received a sceptical response from some public-interest and environmentalist groups. While acknowledging the importance of a more rational approach, these are concerned that events with any danger attached should not be officially labelled as 'safe' — even if the risks are less than one in a million.

But Calman's scheme has, in general, been welcomed by industrial representatives such as those from the pharmaceutical industry, frustrated at what they argue to be the public's inability to distinguish the relative dangers posed by different kinds of risks. Britain's beef industry, for example, has slumped in the wake of the BSE scare, even though only about a dozen cases of CJD have been linked to 'mad cow disease' — in a human population of almost 60 million. □



Calman: tackling risk perception of public.

From solar energy to gamma-ray 'telescope'

Boston. Physicists in the United States and France are planning to convert equipment built to capture solar energy into a giant 'telescope' for detecting gamma rays and making other astronomical observations. The idea was first investigated in the 1970s, but abandoned as impractical.

Researchers working on the Solar Tower Atmospheric Cherenkov Experiment (STACEE) are gearing up to make astronomical observations at the Solar Two power station in Barstow, California, and at Sandia National Laboratory in New Mexico. Similar work is under way in the French Pyrenees at the Cherenkov Low Energy Sampling & Timing Experiment (CELESTE) at the defunct Thémis solar power plant.

Both teams hope to make their first observations in the autumn of 1997. They say that the modified equipment could provide glimpses of an unexplored realm of the electromagnetic spectrum at a cost of less than US\$1 million.

The Solar Two power plant has 2,000 adjustable mirrors or 'heliostats', which can focus light onto a central tower. Scientists have studied the tracking ability of the heliostats, assessed the mirrors' optical qualities, and have measured the night sky background. They detected the flashes of blue Cherenkov light that result when

gamma rays penetrate the atmosphere.

This success was partly due to a suggestion from Tumay Tumer, a physicist at the University of California, Riverside, that a secondary optics system be used to reflect light from each heliostat to a separate photomultiplier detector, reducing background noise. The technique prompted some scientists to reconsider the solar farm approach.

At Sandia — which has 220 heliostats — researchers set up a 3-metre-diameter secondary reflector in August, and demonstrated that they could align their 'telescope' to track stars and spot Cherenkov radiation. The next step will be to attempt to show that lower-energy gamma rays can be detected by increasing the mirror collector area.

Next year, the team hopes to use 50 mirrors at Solar Two to observe the Crab nebula and pulsar, which become visible in October. Rene Ong, the University of Chicago physicist who is leading the STACEE effort with Tumer, says: "That would be the first time light taken from a field of solar reflectors has produced an astronomical image."

The French team, led by Patrick Fleury and Eric Pare of the LPHNE Ecole Polytechnique, also plans to look at the Crab. The project was approved in July by the Institut National de Physique Nucléaire

et de Physique des Particules, and has been awarded \$600,000.

Both groups intend to look at dozens of gamma-ray sources — pulsars, active galactic nuclei (AGN) and supernova remnants — identified by the EGRET instrument on the Compton Gamma Ray Observatory (GRO) using ranks of mirrors that were designed to focus the Sun's rays on a single point at the top of a tower.

The large mirror areas at the Solar Two and Thémis sites should allow examination of a segment of the gamma-ray spectrum, from 20 to 200 GeV, that has been inaccessible to space-borne and ground-based detectors.

Floyd Stecker, a theoretical astrophysicist at the NASA Goddard Space Flight Center in Maryland, says: "These new instruments will look at energies higher than EGRET observed, but lower than those seen from ground-based telescopes like the Whipple Observatory in Arizona."

Stecker hopes the projects will shed light on a class of AGNs called "blazars", which shoot high-energy gamma rays and jets of relativistic plasma towards Earth. "We'd like to know how blazars produce these gamma rays and what kind of new physics might be involved," he says.

If the research proves successful, the US and French groups plan to join forces for ►

NASA asks academy for space priorities

Washington. The US National Aeronautics and Space Administration (NASA) has asked the National Academy of Sciences to assemble an expert group to come up with plans and priorities for space science. The group will present its results to vice-president Al Gore in early December, who will take the recommendations to a 'space summit' later that month at which the White House and Congress will try to agree on funding levels and priorities for the nation's space programme.

Wesley Huntress, head of science at NASA, told the agency's scientific advisory council this week that the interest of the Clinton administration in space science "has increased markedly" since the announcement this summer of possible Martian fossils. But the NASA/academy group will consider more than just plans for Mars. White House science adviser John Gibbons last week charged the experts to look at three broad topics: the Universe; planets; and the evolution of life on Earth and elsewhere.

Tony Reichhardt

Doubts cast on Chernobyl safety claims

Moscow. Yuri Kostenko, Ukraine's minister of environment and nuclear security, became embroiled in new controversy last week when he announced at a press conference in Kiev that the 'fourth unit' of the Chernobyl atomic power station "could blow up any moment", following the detection of a ten-fold increase in radiation inside the sarcophagus.

Kostenko's comments came the day after returning from a meeting of the International Atomic Energy Agency in Vienna, at which he had reported on the state of the Chernobyl power station and stressed the need for Ukraine to receive the ECU118 million (US\$147.5 million) promised by the G7 countries to close the power station. "Any delay may make it impossible for Ukraine to fulfil this task before 2003," he had said a few days previously.

But Evgeny Velikhov, vice-president of the Russian Academy of Sciences, and director of the Kurchatov Institute, claimed that there was "nothing new" in the situation at Chernobyl "except for Kostenko's panic".

Similarly Georgy Kaurov, the head of information at the Russian Ministry of Atomic Energy, said that it was wrong to talk about a possible explosion at the power

station and no data had been recorded on a rise in gamma radiation. Furthermore, the system for monitoring neutrons had been found unreliable during a previous visit by a commission from the ministry five years ago, and no improvements had since been made.

Those who claim that Kostenko's demands are efforts to raise more foreign aid say that their interpretation is confirmed by a chronology of recent events. On the evening of 16 September, several devices installed in the sarcophagus covering the fourth unit registered a rise in the flow of neutrons. Although in principle this could have indicated the initiation of a chain reaction, further measurements indicated that it was most likely the results of faults in the control system caused by heavy rain.

Initially Kiev had kept silent about the incident. But three days later Leonid Kuchma, the president of Ukraine, referred to the incident in discussions with French officials when he said that he was "deeply concerned" about the 200 tonnes of nuclear fuel buried under the power station's sarcophagus. Viktor Baryakhtar, vice-president of the Ukrainian Academy of Sciences, and a nuclear adviser to Kuchma, said that the possibility of a small explosion at Chernobyl "cannot be totally ignored". **Carl Levitin**

US increases health funds but ducks AIDS allocation

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Cause for reflection: the French Thémis plant could be used to detect gamma rays.

► a big experiment, probably at Solar Two. With its 2,000 mirrors, each with an area of 40 square metres, Solar Two has the potential to be "the most powerful and sensitive gamma-ray observatory", says Ong.

But not everyone agrees. Trevor Weekes, head of the gamma-ray astronomy group at the Whipple Observatory, believes that makeshift facilities at solar farms cannot be substituted for fully fledged observatories. He says that the long-term monitoring needed to characterize gamma-ray sources can be done only with a dedicated telescope.

"The solar arrays in Barstow and Thémis have sufficient mirror area to extend ground-based gamma ray detection techniques to lower energies. However, if one were to design an atmospheric Cherenkov telescope from scratch, one would never design the mirrors in this configuration, nor is it likely that one would locate the telescope at these sites."

At best, he says, solar farm instruments can "fill the gap temporarily" before the next-generation space observatory (such as the yet-to-be-approved GLAST telescope) and ground-based observatory (such as the proposed \$10-million ATHENA project at Whipple) become available.

Ong replies: "It could be 10 years before either project comes to fruition, and some interesting science might be done in the meantime." A participant in GLAST, Ong is as anxious as anyone to see the mission fly. But he says the mirrors on the proposed space observatory would not be big enough to detect weak sources. And ATHENA would focus on higher-energy gamma rays than STACEE and CELESTE.

Ong agrees with Weekes that a solar farm 'telescope' will fall short of the "ultimate" gamma-ray observatory. But to build such a mirror from scratch would cost \$100 million.

Steve Nadis

Washington. The budget for the National Institutes of Health (NIH) in the 1997 fiscal year, which began on 1 October, will — as widely anticipated — be 6.9 per cent higher than the 1996 budget, under a bill passed by the US Congress and signed by President Clinton late on Monday. But it has sparked controversy over its failure to specify how much should be spent on AIDS research.

The NIH budget, which will total \$12.7 billion, includes \$90 million to begin the construction of the planned Clinical Research Center on the NIH campus. "I'm extremely pleased," said John Porter (Republican, Illinois), chairman of the House labor, health and human services and education appropriations subcommittee, who authored the 6.9 per cent funding increase and persuaded colleagues to adopt it over the more modest 4.1 per cent increase proposed in the Senate.

The budget law also includes a new compromise on the status of the NIH's politically embattled Office of AIDS Research (OAR). Under this, the office will be allowed to determine how much money is spent on AIDS, to allocate this between the NIH's 24 institutes, divisions and centres, and to shift funds between institutes during the year, within limits. The power to shift this money — up to \$45 million — is a new one, aiming to allow the OAR to respond to changing research developments.

But, reflecting the fact that some House Republicans have opposed earmarking money for AIDS, the measure does not specify how much of the NIH budget should be spent on AIDS research — although this was required by the 1993 law that established the current powers of the OAR. Instead, it says merely that Harold Varmus, the director of NIH, and William Paul, the director of OAR, will jointly agree a figure.

A report accompanying the budget law sets this figure at \$1.5 billion, a 6.6 per cent increase over 1996. "This simply gives [Paul] appropriation authority, without providing a line item that would be disease-specific," said Porter. "It preserves our very strong intent that science determine the allocation of funding for research, and not politics."

Speaking after house passage of the bill, Paul said the NIH would have preferred a single-line item in the budget, known as a 'consolidated appropriation'. But the bill as written nonetheless grants the OAR much of the same flexibility as before, "and in some respects goes beyond" former limits, he says, referring to the power to transfer funds between institutes.

But AIDS activists, the Clinton administration and some scientists called

the compromise a partisan political gambit that ignores the wishes of the scientific community. "The scientists want [the strongest possible OAR]. That's what's so galling about Porter's attitude," said one AIDS scientist.

Gregg Gonsalves, policy director with Treatment Action Group, a national organization that lobbies for AIDS research, said the deal was made "in the best interest of right wing politics". A Clinton administration official who follows the issue admits that "our absolute preference [was] to have the full authority of the OAR", but adds that "we continued to run into a brick wall in the House".

OAR supporters also worry that, if voters return a republican congress in November's elections, the compromise could hurt their position in imminent negotiations to renew the 1993 law that established the OAR as overseer of NIH AIDS research.

Nevertheless the compromise is the closest thing yet to a truce in a political battle which has beset the OAR since 1993, when it was first put in full charge of the AIDS research portfolio. According to one Congressional staff member, neither side can complain too loudly about the arrangement, because it does two things. It allows Porter to say there is no earmark for AIDS in the 1997 spending bill, and it allows Paul to control AIDS funds.

Congressional conservatives have protested that the OAR's control over nearly 12 per cent of the NIH's budget puts AIDS on a pedestal not granted other diseases, at the urging of AIDS activists. But the office is also backed by many scientists, including Nobel laureates David Baltimore of the Massachusetts Institute of Technology, and Torsten Weisel, president of Rockefeller University.

The latter were among more than 350 scientists who signed letters this year to Congressional leaders urging that the OAR keep its full authority. Last March, a committee of 118 experts headed by Arnold Levine, a molecular biologist at Princeton University, recommended that the office should retain its full power over the AIDS purse strings — \$1.4 billion in the 1996 fiscal year.

This summer, the House passed a 1997 spending bill which restricted the OAR's authority, as did the republican-authored law which funded the OAR in the 1996 fiscal year. The parallel Senate bill did not restrict the OAR, but it did not reach a vote. The new bill gives OAR the flexibility to transfer between institutes during the year up to three per cent of a given institute's AIDS budget.

Meredith Wadman

Indian hopes for 'herbal fuel' disappear into thin air

New Delhi. Euphoria over the apparent promise of a 'herbal' petrol made by mixing a plant extract with water is evaporating rapidly (see *Nature* 383, 112; 1996). During a demonstration last week in Madras, Ramar Pillai, the 'inventor' of the technique, failed to produce a single drop of fuel. As a result, India's Department of Science and Technology (DST) has decided not to proceed with its efforts to patent the process.

Valangiman Ramamurti, secretary of the department, says the government is pulling out because Pillai "is not satisfying our evaluation criteria". But he denies that the department had been duped. "I am not saying that," he says, adding that the DST had planned to provide Pillai with all the support he needed — including patent protection — after the process had been scientifically validated.

"The experiment at the Indian Institute of Technology (IIT), Delhi, had raised some questions, and we therefore had it repeated at IIT Madras on 25 September," says Ramamurti. Pillai has blamed the failure of the Madras demonstration on the herbs not having been properly prepared. This excuse has been dismissed by DST. □

Policy on tobacco funds

London. Britain's Cancer Research Campaign (CRC) is to develop a policy with universities and other research organizations to ensure that CRC grants are not linked to tobacco funds. "We believe a code of practice coupled with new research on public attitudes towards tobacco company strategies will influence academic institutions on future deals with controversial partners," says Gordon McVie, director general of CRC. The charity also plans to commission research into public attitudes to the tobacco industry and its influence on health policy in an attempt to dissuade universities from accepting funding from the tobacco industry. □

Iraq plans 'science day'

London. Saddam Hussain, the president of Iraq, has ordered the country's scientists to celebrate a national 'science day' every year on 18 January. The date commemorates the anniversary of the first Iraqi Scud missile attack on Israel during the 1991 Gulf war. It was chosen for its "historical, national and pan-Arab significance", according to an Iraqi radio report. □

Europe's young scientists

Munich. Two young engineering students and a mathematics student, from Germany, the Netherlands and France respectively, were named as Europe's top young scientists last week in an annual competition intended to encourage a new generation of scientists in the European Union. About 12,000 young people between the ages of 15 and 21 took part, and the three winners were each awarded ECU5,000 (US\$6,300).

The jury particularly commended the German winner, 19-year-old Tobias Kippenberg from Bremen, for developing a system for detecting road conditions. He found that dry, wet and icy roads produce different signals when exposed to microwave and infrared radiation, and developed a way for recording surface conditions which, the jury said, could be easily used in cars of the future. □

China acts against polluters

Hong Kong. China's State Environmental Protection Bureau has ordered all registered small factories with serious pollution problems to close from 1 October, a move that — in principle — could lead to the closure of tens of thousands of companies, including small paper mills, tanneries, printing and dyeing factories, oil refineries, pesticide makers and even small-scale producers of radioactive materials.

Although the announcement was officially made last week, some officials had already begun to carry out the instruction. Central Hebei province reported that it had closed 1,500 factories by mid-September and would close 9,000 by the deadline. Most of China's hundreds of thousands of factories operate with little or no waste-control measures. The government has been trying to update its environmental laws, and promised in July to spend US\$20 billion in the next five years to clean up the country. □

Theses on the Internet

Munich. Doctoral students at the University of Chemnitz in east Germany are to be allowed to publish their dissertations on the Internet. In doing so, they will be able to save the cost of printing and binding 50 copies, which can be as high as DM1,500 (US\$1,000), to satisfy German requirements for formal publication. It is not yet clear whether other universities will follow this precedent. □

Lecturers or research students?

London. Britain's Association of University Teachers (AUT) has criticized the practice by which undergraduates are increasingly taught by postgraduate research students instead of full-time faculty members. "Higher education is seeing the emergence of a hybrid part-student, part-teacher," says a survey published by the AUT union last week. "Few have adequate training or departmental support."

Its publication coincided with the launch of a 'concordat' signed jointly by UK universities and research funding organizations, promising to improve the career management of contract research staff. The aims of the agreement include securing employment conditions for contract research staff that are in line with those for permanent employees. □

Nuclear agency opens up

Tokyo. Japan's Atomic Energy Commission (AEC) decided last week to open its nuclear power policy decision-making process to public debate and scrutiny. The move, which is the first of its kind by a government agency in Japan, follows increasing public mistrust in the wake of a sodium leak at the fast breeder reactor Monju last December (see *Nature* 379, 198; 1996) and the rejection, in a local referendum held in August in Maki City, Niigata Prefecture, of plans to build a nuclear power plant in the city.

The commission will make its draft policy documents and expert committees available to the public. It also plans to respond within one month to questions asked by the public in an effort to increase the "accountability" and "public understanding" of Japan's nuclear energy policy, according to Hidenao Nakagawa, Minister of Science and Technology and Chairman of the Atomic Energy Commission. □

Hong Kong seeks 'superchick'

Hong Kong. A four-member research team at Hong Kong University is working to establish genetically the best combination of Chinese and Western chickens to produce a Hong Kong 'superchick', while retaining genetic diversity among Chinese native lines in danger of dying out.

Chinese chickens are renowned for taste and texture, but they grow slowly. This has led farmers to crossbreed them indiscriminately with Western breeds that grow faster, says Daniel Chan, head of zoology at the university and the project leader. The government-funded HK\$8.25-million (US\$1.06-million) project aims to set up a gene bank of the many Chinese pure lines. The project will last three years.

Researchers and the Hong Kong government also hope to stimulate a new export industry in addition to the domestic market of six million consumers of chicken. Breeders and scientists from China are also interested in collaboration. □

Science seeks weapons clean-up role

Cleaning up the legacy of US nuclear weapons production is now the largest environmental project in the world. But science is finding it difficult to contribute to closing the circle opened by the Manhattan Project.

BETWEEN 1945 and 1986, a vast industrial complex in the United States produced 56,475 nuclear warheads. Comparable plants in the former Soviet Union made a similar number. This desperate struggle for nuclear supremacy, in which the two adversaries built 98 per cent of the world's nuclear stockpile, has left a daunting physical legacy.

A huge environmental remediation programme is now under way in the United States to clear that legacy. Citizens' groups and state governments — known in the United States as 'stakeholders' — have demanded immediate and visible action to clean up the mess. But many scientists question the technical basis of the resulting plan. As its costs and drawbacks become apparent, they are arguing for far more basic research now, to support cheaper and more effective clean-up later on.

Jobs by the thousand

When the US Department of Energy (DoE) started to consider the problems in its nuclear weapons complex a decade ago, it found a hornets' nest which, experts said, would take decades and up to \$1,000 billion to clean up using existing technology and approaches. The environmental legacy of the weapons programme in the former Soviet Union has as yet barely been assessed (see page 376).

The US clean-up programme rapidly became little more than an extensive jobs programme. As weapons production ceased, tens of thousands of workers and billions of dollars were transferred into what Alvan Alm, the DoE assistant secretary in charge, describes as "the largest environmental programme in the world in terms of acres, dollars, buildings, whichever way you measure it".

While some of these thousands began to tidy up the mess as best they could, others began work on ambitious and expensive plans for such things as vitrification plant — the construction and operation of which would keep contractors employed well into the next century.

Now, if some optimists are to be believed, the US programme is moving into a new and more constructive phase, in which science could be poised to play a larger role.

Change is being driven by members of Congress and DoE officials who realize that the existing \$6-billion-a-year programme is politically unsustainable, as well as scientists and engineers who question its technical feasibility. Many experts say that such change is badly needed; in the rush to comply with environmental laws and the concerns of local people, they say, the DoE has agreed to approaches for clean-up that are painfully unrealistic.

The most problematic site in the US weapons complex is at Hanford in Washington state, where most of the plutonium for the US stockpile was produced by irradiating and then processing uranium fuel (see page 377). There, a huge volume of mixed liquid waste now kept in underground tanks is eventually to be vitrified and stored elsewhere, at an estimated cost of more than \$30 billion.

According to a study released in January by the National Research Council (NRC), the operating arm of the National Academy of Sciences, the commitment to vitrify "was made with little or no examination of the technical and financial ramifications of the decision". The NRC and other critics say that full recovery of the waste sludge for vitrification may prove impossible with existing technology, and that other options — such as continued use of the tanks until better technology is available, or the permanent disposal of tank contents on site — should have received more thorough consideration.

Other disputed plans for Hanford include extensive reliance on 'pump and treat' of contaminated ground water — the removal and chemical treatment of small amounts of water from vast, contaminated plumes of ground water, which many experts dismiss as useless — and a bizarre proposal to drag old reactor cores weighing thousands of tonnes away from the Columbia river.

Tom Grumbly, who headed DoE's Office of Environmental Management (OEM) until Alm took over in May, and is now under-secretary at the department, says that the need for fundamental research to investigate new science that would eventually support clean-up was clear to him on his arrival at the department in 1993.



Grumbly: backs need for more research.

When the clean-up programme started, Grumbly says, those in charge saw it as "pretty much entirely an applied engineering problem". They were concerned, he says, "that a fundamental research programme would not produce the results that they needed". When OEM was set up in 1989, it simply inherited the staff and multibillion dollar budget previously engaged in weapons production.

As a result, he concedes, "they built a very 'applied' programme, and one which frankly had way too much money going in that they couldn't handle".

Cash for research

Drawing on the advice of his friend Don Kennedy, the ex-president of Stanford University, and others, Grumbly began pushing for a basic research programme worth \$150 million annually to rescue the clean-up programme. But, despite pledging support for the research, Grumbly was unable to find the money to start it. It was finally launched in February, at the insistence of Congress, with a \$50-million budget. In August, the department announced the first round of grants to universities and DoE laboratories.

Just as the programme gets under way, a brand-new, \$230-million laboratory will open this month for chemists and biologists whose research is directed towards DoE's clean-up mission. The Environmental Molecular Science Laboratory (EMSL) at the Pacific Northwest National Laboratory (PNNL), next to the Hanford site, will ►

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Paul Ellis and EMSL's nuclear magnetic resonance spectrometer: going for world's highest resolution.

house a large, multidisciplinary team of DoE scientists and an unmatched array of equipment for use by them and by visiting university scientists.

The new programme and laboratory are supposed to bring first-rate science and new ideas to bear on clean-up for the first time. But a formidable array of barriers that have so far kept science out of the programme will have to be overcome.

First, relations between scientists and the engineering contractors who operate the clean-up programme are not good. "There's a lot of them would like us out there with shovels," says one EMSL chemist of the contractors. Many engineers on the sites view basic scientific research as an indulgence. Investment in research, they correctly surmise, implies that their current approach is inadequate, even misguided. As one Hanford engineer sees it, the research arm of DoE "wants the best science. [It is] not particularly interested in solving anyone's problems."

The tension between the two camps has been exacerbated by less than cordial relations between the Office of Energy Research (OER), DoE's science arm, and the Office of Environmental Management. Mutual mistrust goes back to the foundation of OEM in 1989, when money for science was transferred from OER to the new office, according to some scientists, but then spent on other things. At the launch of the new, jointly administered science programme on 14 February, Grumbly and Martha Krebs, who heads OER, exchanged Valentine cards, implicitly conceding just how bad relations had been.

"There has been very little connection between what OER does and what OEM needs," says Roy Gephart, a geohydrologist at PNNL who has been closely involved with the new science programme. Partly as a result, he says, there has been little flow of new science and technology into the clean-up programme. "Technology transfer is spoken about often, but it is not going on in any effective way."

Pointing to the situation at Hanford, Gephart asks: "What do you see there? \$1 billion has been spent on 'technology development' across the DoE complex, and what do we have to show for it? We should be able to go out on site and see demonstration after demonstration [of new technologies]. But very, very few are occurring."

Gephart believes that, if more money is invested in the right kinds of research, and better links are established to develop the output of that science into solutions, "a whole series of *in situ* technologies that will reduce costs and risks" associated with waste on the DoE sites could result.

These would include bioremediation, using enzymes to attack groundwater contamination; chemical treatments to convert mobile, soluble ions of toxic elements into non-soluble ones which precipitate out and stay put; and development (to page 379) ►

Russian waste goes to ground

Washington. On 29 September 1957, a vast explosion rocked the main Soviet plutonium production facility near the city of Chelyabinsk in western Siberia. The blast, which was equivalent to 100 tonnes of TNT and released two million curies of radiation over a hundred-mile tract of neighbouring farmland, was caused by the overheating of a mixture

doing in clean-up, they don't have a programme," says Norris.

In the early and very desperate days of the Soviet weapons programme, high-level waste was drained straight into the Techa River at Mayak. But this had stopped by 1953, and the waste was put in tanks, and later dumped in nearby Lake Karachai. "They weren't totally

careless," says Norris. "They knew that they were handling toxic and dangerous materials." But the measures taken to protect the population from these materials were, by Western standards, minimal.

Work started quite early on vitrification of the tank waste; pilot plant was built in the 1970s, followed by full-scale plant which has turned 250 million curies — one-quarter of Mayak's tank waste — into glass



Russia's weapons complex in western Siberia harbours far greater clean-up problems than US sites.

of sodium nitrate and sodium acetate in a waste tank.

For both the Soviet Union and the United States — which learned of the accident through its espionage network, but released no information to the public — the explosion served as a timely warning of both the volatility and potential danger of high-level nuclear waste.

The Soviet weapons complex, built hurriedly by Stalin and his secret police chief, Lavrenti Beria, has many features in common with that of the United States. Plutonium was produced at three sites in western Siberia — first at Mayak near Chelyabinsk, and later at Tomsk and Krasnoyarsk (see figure).

Radiation released

But, according to scientists in both countries, the scale of the environmental crisis left behind with the ending of weapons production is far greater in the Soviet complex, with perhaps 100 times as much radioactivity released from the sites. "It's far, far worse than what the United States did," says Stan Norris, a nuclear weapons expert at the Natural Resource Defense Council in Washington DC, of radiation releases that were allowed to take place in the Soviet complex during weapons production.

Unlike the United States, which closed the last of the reactors used to produce plutonium for weapons in 1988, Russia is still operating several of its reactors — which also generate electricity — and is not spending billions of dollars a year on environmental remediation. "In terms of what we're

for temporary storage above ground.

Contaminated lakes and rivers around Mayak have been receiving political attention for some time. The previous president, Mikhail Gorbachev, for example, set up a commission to examine problems at the site, and special laws have since been enacted to protect the environment there. Efforts are continuing to stabilize radioactive contamination in Lake Karachai, using concrete blocks to hold contaminated mud in place.

At the two other plutonium production facilities in Russia — further east, and away from centres of population — high-level waste is disposed of by being pumped directly into deep wells. This practice, which would be politically as well as legally unacceptable in the United States, has been used to dispose of around 2 billion curies of radioactive waste at the two sites — ten times more than the waste inventory of the Hanford tanks, and almost 1,000 times as much as has been released into the environment by the entire US complex.

Russian scientists have defended deep-well disposal, arguing that the waste will decay before it escapes. But public protest halted a plan for deep-well injection at Krasnoyarsk in 1989.

Minatom, Russia's nuclear agency, has signed an agreement with the US Department of Energy to share waste management and clean-up technologies. But according to Alexei Yablokov, director of the Centre for Environmental Policy, an independent group in Moscow, the situation in the Soviet complex is continuing to deteriorate.

C. M.

Hanford faces up to its nuclear hangover

Richland, Washington. The Hanford Engineering Works in Washington state, as the most notorious environmental wasteland in the United States, has an infamous reputation to live down to. But in reality, the most striking facet of the 530-square-mile site is its serenity.

The blue-collar members of the 12,000-strong Hanford workforce are out in the sunshine, tidying up. The main processing buildings — disused since Hanford withdrew from its role in the nuclear weapons programme in 1990 — are being methodically stripped down to a condition in which they will remain contaminated, but can be cheaply maintained.

Contamination from Hanford ground water puts 6,000 curies of radiation into the Columbia river each year — about the same as it brings down from lands upstate and in Canada. The Hanford tanks are hidden underground, but neatly labelled.

Plutonium production — Hanford's main task during the Cold War — produced large volumes of radionuclides dissolved in nitric acid. The much smaller weapons programmes of Britain and France wisely kept such acid in stainless-steel tanks. But the Hanford engineers chose to turn the acid into alkali so that it could be kept in cheaper, carbon-steel tanks. They have been struggling with the consequences ever since. The tanks are Hanford's greatest headache: 177 giant underground vestibules filled with a varied and highly radioactive cocktail of liquid, paste and precipitate.

Many of the 149 older tanks, which have just a single shell of steel, have leaked at some stage, losing perhaps one million gallons of waste.

Ferrocyanide — a compound with an unfortunate tendency to explode at high temperature — was added to remove radioactive solids by precipitation so that less radioactive liquid could be dumped in the soil. Contents were mixed in unknown ways as waste was transferred from tank to tank. Chemical reactions cause some tanks to bubble hydrogen and other gases, and make it hard to keep track of the contents.

Not so scary

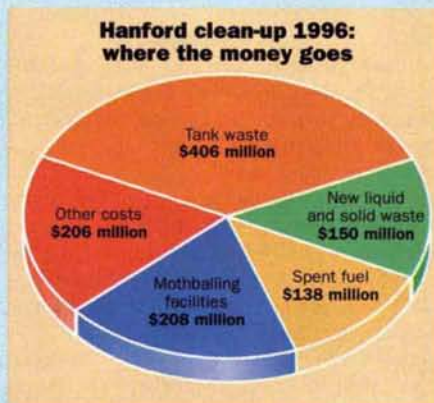
But the situation in the tanks is not quite as scary as it sounds. All the liquid waste is due to be moved to the 28 newer, double-shell tanks (leaving behind precipitate and sludge) which have no history of leaks. According to Ron Lerch, deputy director of tank remediation at Hanford, the ferrocyanide has decomposed completely in some — maybe all — of the tanks to which it was added.

Furthermore, with 99 per cent of the 200 million curies of radioactivity in the tanks coming from short-lived isotopes of cae-

sium and strontium, they will lose half of their potency in 30 years. (In 800 years, only one curie from these two isotopes — plus 125,000 curies from other, long-lived radionuclides — would remain.)

Another pressing issue on the site is the fate of 2,000 tonnes of spent nuclear fuel now corroding gently away in basins of water next to one of the disused reactors by the Columbia river. Under an accelerated \$700-million programme, the fuel will be removed at the end of next year, dried off and stored in a 'temporary' vault.

Another accelerated programme will



mothball B Plant, one of the five huge canyon buildings at Hanford. The mothballing will be complete two years from now, and will cut the annual cost of maintenance from \$20 million to \$1 million.

But 150 million curies of extracted caesium and strontium and their waste products will remain next door in pools — from which the intense radiation emits an eerie, blue light — and in need of constant attention. Still more is stored in organic solvents. And three-quarters of a million curies lurk in several large air filters. "We don't know what to do with them," concedes Bob Heineman, B Plant manager. Four other canyons at Hanford house similar problems, exacerbated by the presence of large amounts of plutonium.

Although many question its efficiency, a good deal of technically straightforward clean-up is therefore now taking place. It is true that no radionuclides are leaving the site. But progress is being made in containing what is already there. The real crisis concerns not the present but the future.

Under the 1993 version of the so-called Tri-party Agreement between the Department of Energy (DoE), Environmental Protection Agency and Washington state, the department is obliged to pursue expensive and technically dubious clean-up strategies.

Top of the list is the proposed vitrification of the entire contents of the tanks, for underground storage off the site and out of the state. The National Research Council

(NRC) has said that there is neither a technical nor a financial basis for this decision.

Three problems stand out. It is not known whether 99 per cent of the waste can be retrieved from the sludge-filled tanks, as promised. How much of the retrieved waste would be suitable for vitrification is also unknown. And there is nowhere to put the resulting glass: even if planned underground stores are built, they would not be large enough for all the Hanford waste.

But vitrification is politically attractive, because it creates jobs, meets regulatory requirements and shows that something is being done. Many of the 'stakeholders' are tired of endless study of the problem, and want to see 'action'. Whenever the DoE talks about research, says Don Wodrich of its Richland office, "the stakeholders say 'we've been hearing that for years'". He adds: "Until we start vitrifying some of the tank waste, I don't think we can have meaningful discussions about the rest."

Demonstration plants

In April, the DoE published an environmental impact statement, confirming its decision to build two 'demonstration' vitrification plants at Hanford to process low-level waste from the tanks, and a full-scale plant to vitrify the rest. The cost was estimated at between \$32 billion and \$42 billion. Many scientists hope that fiscal reality and technical infeasibility will squeeze this plan out of existence. Last month, an NRC panel recommended that it be shelved.

The Tri-party Agreement also calls for extensive 'pump and treat' of groundwater at Hanford — a strategy whose main advantage is psychological (see page 378). As for the defunct reactors by the river, the state and the stakeholders want them away from the water table. The plan is to dig up each reactor core, encased in 15,000 tonnes of concrete, and drag it uphill.

This bold plan of action calls for four transporters each able to carry 16,400 tonnes — twice the capacity of the largest such cart in the world, used by NASA for space shuttle launches — and the construction of a road 25 miles long that can carry such a vehicle, to lift the blocks 140 ft up to the dumping ground a couple of miles away at a shallow gradient. A report prepared for the US Senate called the scope and uncertainty of this proposal "staggering".

Tom Grumbly, the under-secretary for energy, points out that the department had to reach agreements, such as that at Hanford, in a hurry to fend off legal action. "There's going to be plenty of room for us to change strategy as we move forward," he says. "But one of the big criticisms of the programme is that it has stood in place too long. We need to get going."

C. M.

Research could realize elusive goals

Richland, Washington. If clean-up of Hanford and the other nuclear weapons production sites were to proceed using existing technologies, most experts believe that the task could continue indefinitely without success ever being achieved.

That is because some problems on the sites — in particular the ground contamination caused by past leakages of toxins and radionuclides — simply cannot be fixed using available technology. Others, such as the need to dispose of large volumes of highly radioactive mixed wastes, can be handled with existing technology, but only at extraordinarily high cost.

Many scientists believe that more basic research could provide the progress that would break this impasse. Of the many areas where progress is needed, four demand particular attention.

The first is bioremediation, which would employ natural enzymes in the rock or soil to break down and destroy organic compounds, or to immobilize radionuclides and toxins. Such techniques can be shown to work in the laboratory, but obstacles remain to their successful application in the field.

Another potential means of stopping radioactive contamination is chemical attack by reducing or oxidizing agents — known as redox manipulation — to convert soluble (and therefore mobile) contaminants

into insoluble forms that will stay put. Both redox manipulation and bioremediation offer the prospect of reliably containing nuclear contamination. Their successful application might remove the need for extra-



Madia: excited by success with grants.

ordinarily expensive action to remove the contamination from the weapons sites.

A separate area of chemistry affecting clean-up is the study of the mixed liquid wastes, which must be better understood if they are to be processed and vitrified at reasonable cost. The cost of vitrification is closely tied to the volume of glass to be created and stored (for regulatory reasons, the cost of its long-term storage is extraordinarily high). Chemists believe that careful study of glass structures and of the waste itself could lead to far less expensive vitrification options.

Finally, research is needed to improve the characterization not only of the problem itself — especially the condition and spread of underground plumes of waste — but also of the associated health and environmental risks.

University and government scientists were awarded 138 grants in August under the DoE's new Environmental Management Science Programme to support work in all these areas.

The programme is not the first in the United States to tackle some of these problems — there is already a bioremediation programme in the Office of Energy Research, for example, and programmes in environmental geochemistry at the National Science Foundation. It is, however, the first to be directed specifically at DoE clean-up.

Reassuring scientists

But according to Kevin Crowley, director of the National Research Council (NRC)'s board on radioactive waste management, many DoE officials still oppose the research programme.

To reassure participating university scientists that it will actually survive, the department has taken the unusual step — recommended by the NRC — of awarding their full, three-year grants straight away, so that they know that their work will be completed whatever happens to budgets. The programme, according to Hazel O'Leary, the energy secretary, will "begin to assure the careers of some of the scientists who bring their expertise to the table".

Ten of the new grants — worth \$16 million over three years — went to the Pacific Northwest National Laboratory (PNNL), the DoE laboratory managed by Battelle at the Hanford site.

Bill Madia, director of PNNL, says he is thrilled with the laboratory's success in the competition. But anything less would have been a grave disappointment — for the energy department has just spent \$230 million on a brand-new laboratory there, purpose built to carry out precisely the kind of science that the new programme supports.

The new Environmental Molecular Sciences Laboratory (EMSL) will officially open this month, although the laboratory has been steadily hiring staff for three years. It represents a new concept for the DoE — a multidisciplinary laboratory, built to conduct basic research directed towards the department's environmental mission.

Thom Dunning, director of EMSL and formerly a computational chemist at the Argonne National Laboratory in Illinois, wants to make science an integral part of the clean-up programme, instead of just a 'job-shop' to which the engineers can turn in an emergency.

"There's still not a good understanding of what science has to offer" to the programme, he says. "Over the next few

years, I hope that its importance will become clearer to our colleagues."

One hundred and twenty scientists and 80 postdoctoral students are already working at EMSL. One of them, Sunney Xie, a 34-year-old chemical physicist who joined EMSL from the University of Chicago, is investigating how near-field scanning optical microscopy can be used to image single molecules. The technique has many potential applications, but at EMSL interest is focused on how it might be used to observe the action of enzymes

IMAGE
UNAVAILABLE
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REASONS

Fuel elements corrode after 20 years in the water-filled basin at Hanford.

that could be used in the bioremediation. The technique could be used to make images of enzyme reactions, Xie says, assisting in their characterization.

A major focus of activity at EMSL is the use of nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) to characterize materials and processes. The new laboratory building will house very powerful equipment for doing this, including an NMR machine with a \$7-million superconducting magnet and a minimum frequency of 900 MHz. This will give it the highest field, and therefore the highest resolution, of any such machine in the world, says Paul Ellis, an associate director of EMSL.

Understanding glass structure

The equipment will, among other things, enable scientists better to characterize the glass forms for waste vitrification. "It's very important to understand what limits the concentration of materials in the glass," says Ellis. The solubility, or amount of waste that can be accommodated in a given volume of glass, is limited by the latter's composition. But, Ellis says, "you don't have any idea what's going on, you just know you can't put any more [waste] in". With better understanding of glass structure, it should be

possible to optimize glass formulation to accommodate far more waste.

Such optimization, coupled with improved waste-separation processes before vitrification, could have very positive consequences for nuclear clean-up, EMSL scientists say.

Hanford vitrification costs alone have been estimated at \$30 billion or more; Dunning says that fresh approaches based on new science could cut this to as little as \$2 billion.

Ellis also points to the need for better catalytic processes, to enable the removal of nitrates from the tank waste before vitrification. The amount of nitrates in the tanks would generate 25,000 tonnes of nitrous oxide emissions if the waste was processed for vitrification with existing techniques, he points out — but the Clean Air Act allows only 40 tonnes of such emissions each year from any given plant. New and highly efficient catalytic processes are needed to reduce the nitrates, Ellis says, if the waste is to be treated in a reasonable time-frame.

Effective enzymes

Magnetic resonance is also being used at EMSL to study enzymes for bioremediation of trichloroethylene and tetrachloroethylene, both troublesome (although nonradioactive) ground contaminants on the weapons sites. Known enzymes have not lived up to their promise — largely because the process that breaks down the pollutant produces intermediates that poison the enzyme. Study of the structural chemistry of the enzymes could lead to the discovery of more effective ones that will not poison themselves.

Related work deals with enzymes that could prevent the underground spread of toxic metals and radionuclides by reducing them to insoluble states. Such bioremediation is arguably the 'holy grail' of the clean-up programme; it is unproven on a practical scale, but its potential to transform the entire multibillion-dollar programme remains beguiling.

Nothing sums up the gulf between scientific potential and current practice better than the contrast between the potential of bioremediation to clean up waste *in situ*, and the current practice of 'pump and treat', which critics say achieves little more than the illusion of activity.

"You see a substantial amount of pump and treat on the sites, which everyone agrees is ineffective in actually solving the problem," says Madia. Given the constraints of time and money on the programme, he says, "project managers are quickly realizing that they can't get there with the best available technology". Clean-up goals will not be met, he says, "unless we get on with bioremediation" and other new techniques. C. M.

► (from page 376) of effective underground barriers. The same principle applies to vitrification chemistry: more sophisticated approaches could drastically reduce the volume of glass produced, and its associated costs and storage problems.

But, even if the research was carried out the technology development chain repaired, major institutional, legal and political barriers would remain to the implementation of a sounder, science-based clean-up programme. The NRC, in its January report *Barriers to Science*, describes "a common pattern of behaviour" inside the DoE Office of Environmental

IMAGE
UNAVAILABLE
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REASONS

US Department of Energy

Outdated technology? Hanford workers use backhoes to dig up old drums of hexone.

Remediation, a large component part of OEM, under which "what happens is driven too often by the internal needs of the organizations charged with the remediation work, rather than by the overall goal".

Grim picture

The NRC paints a grim picture of a programme driven almost entirely by a combination of these internal needs and immediate regulatory requirements. It points out that the department has sometimes brought these regulations on itself by succumbing to political pressure to reach agreements with the states "without adequately considering technical feasibility, cost, or schedule". The result, it says, is that "the highly talented, dedicated and experienced people" working on the programme "share the sense of frustration felt by the committee and the public". Its worst characteristics, it says, "are in part responses to the pressures of doing science and engineering in a political fish bowl".

Grumbly has certainly felt the full force of these pressures. At a hearing in March before the Senate Armed Services Committee to confirm Grumbly's promotion, Strom Thurmond (Republican, South Carolina), the committee chairman, was less concerned about the appliance of science than about 2,000 proposed job losses at Savannah River — the second-worst site in the weapons complex — in his home state.

As the 93-year-old senator railed at him to "reassess" the job losses, Grumbly was left in no position to talk about research.

"We've got the message that real clean-up is what the Congress will support," he said.

Such pressure from Congress and the public for "real clean-up" — involving plenty of federal jobs — does not augur well either for science or for a long-term solution to the mess. Instead, it may pull the programme in the opposite direction.

On 14 February, Grumbly said that the science programme would yield results "in eight to ten years". But this summer, shortly after his appointment to Grumbly's old position, Alm announced an ambitious programme to complete large parts of the clean-up within ten years, leaving precious little time for science to have an impact. The precise meaning of Alm's deadline remains unclear: each site is now drawing up new plans to conform with it.

Alm and Hazel O'Leary, the energy secretary, defended the ten-year plan when they announced the first grant winners under the new Environmental Management Science Programme on 20 August, and denied that these two priorities conflict. "You never know how high you can jump if you haven't got a bar," says Alm. The science programme "will give us the technologies we need during the ten-year period, and a stronger base for the more intractable problems of the future".

Careful management by the DoE and the Congress could prove Alm right. The department could begin by doing the easy tasks, such as decommissioning buildings and shifting waste from unstable, temporary storage, while scientists occupy themselves with the research that might yield feasible solutions for the more brutal problems, such as contaminated ground water and the contents of the Hanford tanks.

Cynics doubt that this will happen. They predict that the public will ask for — and get — action, of sorts, with most of the money continuing to be spent on endless rounds of studies and plans, and on extravagant remediation schemes of dubious worth. As Mike Knotek, associate director of PNNL, puts it, "the regulators and the stakeholders are very reluctant to allow the DoE to research the problem".

But there is now a real chance that the shortage of money will break this pattern. Over the past year, Knotek has detected a change in the approach of Congress and the DoE. "Forcing a budget cap on things has probably helped," he says. "It has forced people to see that science is critical to making things happen."

"Congress is already telling DoE that we're not going to be able to afford these lavish plans it has to clean up the sites," says Thom Dunning, the director of EMSL, referring to vitrification of the tank waste and other expensive proposals for the weapons complex. Gephardt adds: "We're now entering a period in which people realize that resources and technologies are limited. That should leave science in a very good position."

Colin Macilwain

Concentrating the mind

SIR — Your News story (*Nature* **382**, 657; 1996) about the ethical propriety of allowing a man about to be executed (Joseph Paul Jernigan) to donate his body to science (whence it was meticulously sectioned and recorded) provided a discourse worthy of mediaeval scholastics.

The Center for Bioethics thought there was a "possibility of coercion". How do you threaten a condemned man? With eternal damnation?

The lawyer thought it a bad example — future prisoners might find the idea so attractive that they would neglect to appeal. I have known many dedicated scientists, but never any that committed.

Marcelle LaFollette thought that, had Jernigan known, he might have objected to being broadcast on the Internet. How many of us will come that close to immortality?

The Legal Defense Fund thinks it a bad precedent because future donations might be abused by "prison officials". How? Kept in the fridge? Black-market organ sales?

And, finally, the same fund objects that the informed consent was tainted: "you're hardly in the frame of mind to make some sort of long-term judgement" when on death row. But, if not when facing eternity, when?

A pity you didn't consult Dr Samuel Johnson, who wrote: "When a man knows he is to die in a fortnight, it concentrates his mind wonderfully."

Stuart Martin

*Mt Mansfield Television Inc./WCAX-TV,
PO Box 608,
Burlington, Vermont 05402, USA*

SIR — I wish to support those who accept as ethical the 'visible man' and 'visible woman' anatomical displays on the Internet.

I do not support capital punishment. But I respect scientific ideals and ethics. I hereby freely declare that, if I were put on Death Row and executed, or killed in any other way, I would passionately wish my body to be used for any reputable scientific purpose. 'Reputable' includes respect for the principle that scientific knowledge is public knowledge. I hereby declare that I would be happy for my anatomy to be made available on the Internet.

A signed copy of this letter has been deposited with my personal solicitor.

Michael McIntyre

*Centre for Atmospheric Science,
Department of Applied Mathematics
and Theoretical Physics,
University of Cambridge,
Cambridge CB3 9EW, UK
e-mail: em@damtp.cam.ac.uk
http://www.damtp.cam.ac.uk/
atmos-dynamics*

SIR — Although Meredith Wadman says of the 'Visible Human' that "the value of [this] anatomical database is undisputed", I would like to suggest that its worth has yet to be established. The fact that 600 institutions and individuals have licensed this database is hardly evidence of its worth. People download gigabytes of garbage from the Internet every day.

I am not saying that the 'Visual Human' is garbage. I just wonder what it has taught us that we didn't already know from the many beautifully detailed anatomical atlases and surgical texts produced over the past century.

Richard Wassersug

*Department of Anatomy and Neurobiology,
Sir Charles Tupper Medical Building,
Dalhousie University,
Halifax, Nova Scotia B3H 4H7,
Canada
e-mail: tadpole@is.dal.ca*

Unscientific speculation

SIR — David Swinbanks reported recently on the threat to public health in Japan caused by a massive outbreak of *Escherichia coli* 0157 enterocolitis in Sakai city, near Osaka (*Nature* **382**, 567; 1996).

The health ministry named white radish sprouts as a likely source of the infection, but no evidence could be produced to support this hypothesis. Furthermore, the normal procedure in such circumstances of carrying out a case-control study, used recently, for example, to explain an outbreak of food poisoning through beef products in the United States, seems not to have been followed. Unscientific speculation and premature disclosure seem to put in doubt the reliability of the Japanese health ministry on such matters.

Yasuhiro Tokuda

*Okinawa Chubu Hospital,
Gushikawa City,
Okinawa, Japan*

Logical conclusion

SIR — Edwards¹ and later correspondents do not criticize Beck-Bornholdt and Dubben's interesting remarks² about logic severely enough: the issue they discuss is central to the truth of quantitative statements. A British minister recently claimed, with the safety of eating beef in mind, that scientists mean something different by the word risk from what other people mean, rather than merely tending to quantify it more precisely.

Beck-Bornholdt and Dubben make two mistakes. The first is that the validity of syllogistic reasoning depends on the truth or certainty of the premises. A deductive argument is, as an argument, just as valid if it has false premises, let alone premises of low probability. Second, the inference about the Pope is simply a quantification mistake between 'any given human being' and 'any human being at all': more precisely, using the received conventions of elementary logic, between

$(\forall x) \neg HBx \rightarrow \{P(x = \text{the Pope}) < 1\}$
which is true, and

$P[(\forall x) \{ (x = \text{the Pope}) \rightarrow HBx \}] < 1$
which is false.

So it seems the methods of statistical inference, and the Pope, can sleep easily in their beds.

John Godfrey

*41 Lawford Road,
London NW5 2LG, UK*

1. Edwards, A. W. F. *Nature* **382**, 102 (1996).

2. Beck-Bornholdt, H. P. & Dubben, H. H. *Nature* **381**, 730 (1996).

● This correspondence is now closed — Editor, *Nature*. □

Storm in a teacup

SIR — One of the many reasons I enjoy reading *Nature* is for the snippets of information about the 'classics' that come one's way: I learned, for example, of the source words in Greek for tectonics (**382**, 392; 1996), as also about the brilliant Michael Faraday's pioneering work on metal colloids (**382**, 581; 1996).

But I read with surprise the attribution to Caliban of the quotation "O brave new world that has such people in't" in Ryan Huxtable's review of *The True History of Chocolate* (**382**, 411; 1996). The Bard puts these words in Miranda's mouth in Act V, Scene i, of *The Tempest*.

Ajoy Baki

*Department of Geology and Geophysics,
Louisiana State University,
Baton Rouge, Louisiana 70803, USA*

Train of thought

SIR — Reading Philip Anderson's obituary of Nevill (no 'e') Mott (*Nature* **383**, 121; 1996) reminded me of an anecdote I heard some 25 years ago.

Mott was travelling on the Paddington to Bristol train when three thoughts occurred to him. First, he was no longer at the physics department at Bristol but Cavendish Professor at Cambridge; second, he had travelled to London earlier that day by car; and third, he had been accompanied by his wife.

Michael Rodgers

*Spektrum Academic Publishing,
33 Beaumont Street,
Oxford OX1 2PF, UK*

International PhD standards

SIR — I recently watched from the sidelines as the university committee charged with administering PhD degrees resolved disagreement by external referees on the worth of a PhD thesis submitted by a student of mine.

One reviewer (from the United States) claimed the thesis provided insufficient advances in the field of study and declined support for the award of the degree, another (from the United Kingdom) judged the thesis excellent and requiring only minor amendments. The process required the judgement of a third external referee who returned a positive grading. The process prolonged the examination period, putting extra pressure on the candidate and raising the question of the nature of international standards for this type of degree.

The extremes were, I believe, symptomatic of different expectations by reviewers of acceptable thesis content — expectations that may reflect quite different perceptions of just what constitutes a PhD degree today. These may largely be related to the period expected for completion — in the United States often 5–6 years, in the United Kingdom up to 3 years. Some European countries expect up to 6 years. The trend in New Zealand is to require com-

pletion within 4 years.

As any biological scientist knows, the product of 6 years' work is vastly different in scale from what may be achieved in 3 years. There is also a greater likelihood of published products within the longer time — a requirement of our negative examiner to obviate his concerns but one that would have prolonged the examination process by many months. Different international expectations must affect the external reviewing process used by most universities to protect the integrity of their higher degrees.

Framing the PhD study to be conservative and guaranteed to yield desired results; directing the course of research to avoid difficult or more challenging areas as they arise; encouraging early publication of any completed work or even incomplete work and selecting external reviewers on the basis of certainty ('cronyism') rather than risk a result based on open expectation — these are all strategies available to supervisory panels to counter possible criticism of thesis content, but most of them are surely contrary to the spirit of investigative science.

Operating longer PhD programmes best suits the universal PhD prescription requirements which require evidence of

independent research and advance of knowledge, but is this the best way to encourage students into advanced science? A 6-year period may better suit the generation of results suitable for publication by the candidate (and grant-dependent supervisors), but it is surely too long as an apprenticeship for a science career. If the PhD degree is to be truly international as a qualification for postdoctoral, academic and industrial positions, reasonable boundaries are needed for the demonstration of both independence in research and advance of knowledge.

David W. Fountain

*Department of Plant
Biology and Biotechnology,
Massey University,
Palmerston North,
New Zealand*

Proving difficult

SIR — An intelligent observer, conscious of the guiding principle of science that the crucial means to distinguish truth from falsehood are those of experiment, but unfamiliar with the notion that paranormal phenomena are 'impossible', would raise his eyebrows on coming upon the following assertions, made recently by two supposed-ly reputable scientists:

(1) Crane, in reviewing Nicholas

So far, systems in the ÄKTA design family include:

- ÄKTAexplorer, for method development and scale up of every biomolecule
- the new ÄKTApurifier, for purification of peptides, oligonucleotides and other biomolecules

Humphrey's book *Soul-Searching (Leaps of Faith in the United States)*¹, suggests that we can save ourselves the trouble of looking at claims of the paranormal by invoking Hume's argument that it is more reasonable to believe that human error lies behind such claims than it is to "believe that some fundamental law of nature has been disrupted".

(2) Hyman, for the purposes of dismissing apparently strong evidence for a 'remote viewing' capability^{2,3}, asserts somewhat similarly that no matter how many investigations of the paranormal, carried out by whatever means, yield positive results, there will still be no proof that the alleged phenomena occur.

In response to enquiries as to why the usual mechanisms of science should be abandoned in this special context, our observer would be directed to study *Soul-Searching* in order to understand why claims of the paranormal are not taken seriously by scientists. But a subversive parapsychologist would suggest looking also at my review⁴, whereupon our friend would realize that Humphrey's arguments are flawed and hence of no value. He would study also some of the original research⁵⁻⁷, and wonder whether the scientists might not be making a monumental error in condemning it so vehemently.

The fact that scientists at large do not come to the same conclusions as our mythi-

cal observer stems, I believe, from two main factors, whose existence mocks the claim of science to be the agent of unveiling the truth, however strange that truth may appear: 'received knowledge', reinforced by the activities of propagandists; and the publishing policies of journals, which limit very effectively the acquaintance that the ordinary scientist has with parapsychological research, and thereby make informed assessment of the work in general effectively impossible.

The references below will provide readers with a better perspective with which to evaluate the evidence. And, for the benefit of those with access to the World-Wide Web, I have created a page, located at <http://www.tcm.phy.cam.ac.uk/~bdj10/psi.html>, with links to the text of some of these and to sites where more information may be obtained.

Brian D. Josephson
*Cavendish Laboratory,
 University of Cambridge,
 Madingley Road,
 Cambridge CB3 0HE, UK*

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True but strange?

SIR — Ernst *et al.* (*Nature* **381**, 361; 1996), in writing about complementary medicine, do not reflect the true results achieved with homeopathy.

For example, initial aggravation in a homeopathic case is the optimal reaction that can be expected from a correct constitutional remedy. Mental effects, if they are not qualified, mean nothing. For instance, if depression changes into irritability, that is a good sign and a prognostication that a cure will eventually ensue. And Ernst *et al.* do not differentiate between classical and 'mongrel' homeopathy, which is giving mixtures of remedies repeatedly for the disease and not for the patient.

I agree that "such survey data are inevitably limited". For instance, aggravation may occur because of wrong homeopathic prescribing, when a lot of remedies mixed together are given repeatedly. But there are also curative reactions — apparent aggravations — that foretell a complete cure with correct prescription as in classical homeopathy with one remedy and one dose only.

George Vithoulkas
*International Academy
 of Classical Homeopathy,
 1 Perikleous str,
 Maroussi,
 Athens 15, Greece*

ÄKTAdesign: an open purification platform for all of your biomolecules

What type of purification is going on in your lab? Do some of your colleagues develop methods and optimize schemes to purify peptides, proteins, or oligonucleotides at every purification scale? Are others purifying natural, synthetic and recombinant peptides? Are yet others purifying native or recombinant proteins? Or perhaps you do all of this yourself.

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Search strategies the answer

SIR — Koreaki Ito (*Nature* **382**, 666; 1996) recommends the abandonment of British in favour/favor of US spellings in order to make more efficient the automated searching of on-line databases. Dangerous waters indeed!

Some years ago, as scientific editor of *The Canadian Entomologist*, one of the oldest scientific journals in North America, I made a similar suggestion. Ambiguously and awkwardly stuck here between the Empire and the rebel Colony, we Canadians had compromised in that oh-so-typical Canadian fashion — the journal permits either spelling convention. That not only made for a lot of work for the copy editor, it was also an insurmountable challenge for many authors, who illogically used both spelling conventions in single manuscripts. However, my suggestion to adopt a single (American) spelling convention in order to reduce this confusion was met with howls and screams of protest. I was called some pretty nasty names and I beat a hasty retreat. Evidently, even scientists cannot be counted upon to treat the mechanics and symbols of language objectively — and I would predict that UK editors who might recommend a similar change would receive pretty much the vituperation I endured.

My personal databases use a single spelling convention — American. But the solution, surely, for the public databases, is simply more intelligent programming. There aren't a lot of words that have dual spellings and it would be a simple matter to have a look-up table to ensure that a search for 'behavior*', for example, would also automatically search for 'behaviour*'. Such a scheme, intelligently constructed, could probably automatically find up to 90% of cases of alternative spellings.

Stephen M. Smith

Department of Biology,
University of Waterloo,
Waterloo, Ontario, Canada N2L 3G1
e-mail: bugman@sciborg.uwaterloo.ca

SIR — Some weeks ago I searched for information about Münchhausen's syndrome in the Medline Database 1991–95. The entry 'Münchhausen' is not possible because the system does not accept the German letter 'ü'. The correct transliteration to 'Muenchhausen' yielded no results, but the version 'Munchhausen' led to 26 entries. After omitting the second 'h', however, I found 283 entries under 'Munchausen'. But in this form the name is badly mutilated for those who speak German and are familiar with Baron von Münchhausen's name and tales. I cannot understand why not even personal names are spelt correctly in such a database.

This is not the only example. The name of Gottfried Ritter von Rittershain (paediatrician 1820–83) is cruelly shortened in Ritter's syndrome, and that of Georges E. A. B.

Gilles de la Tourette (internist, 1857–1904) in Tourette syndrome, or sometimes Gilles-de-la-Tourette syndrome. Moreover, I found the name of Kurt Gödel (mathematician, 1906–78) shown as 'Godel' in *The Lancet* (**346**, 1172; 1995).

Such discrepancies are also found in scientific names. The name of the genus *Necrophorus* (burying beetles) was first published as *Nicrophorus* because of a typographical error. Although the author, Fabricius, corrected the error as early as 1801, both forms appear in the *Biological Abstracts* database.

It is unlikely that authors and editors of English-language journals will agree to uniform spellings. The best solution is a more intelligent searching system for databases that will offer alternative spellings where they exist.

Achim Th. Schäfer

Institut für Rechtsmedizin,
52057 Aachen, Germany

SIR — While I have the greatest sympathy for Koreaki Ito when faced with the vagaries of spelling in the English language, I feel compelled to comment on the techniques used in his searches.

The method used was a textword search in an individual field. In general, this is the most inefficient way of searching any database. The subject thesaurus system adopted by most text databases was designed to alleviate the very problems caused by differing use of terminology and variations in spelling. A subject search using the Medline index term 'disulfides' would have found all articles on this subject regardless of the spelling convention used.

If a searcher insists on looking for a specific word in any one field, most databases will accept some form of truncation symbol. Thus, in some Medline systems, a search for the term 'disul-' will detect any words beginning with that series of letters. In some of the better quality databases, truncation is also possible in the middle of words, so a search on 'col*r' would detect both 'colour' and 'color'.

Indeed, there are many pitfalls associated with textword searching. In many databases, the search run on the word 'disulfide' would not find the plural 'disulfides'. In addition, no amount of regulation would do away with variation in spelling due to typographical errors, which can slip through the editorial net both in the original journal and at a later stage when the details are entered onto the database. The subject search method can help to minimize the effect of human error.

This whole scenario highlights one of the problems created by the brave new world of the Internet. The information on any database is of value only when it can be accessed efficiently and effectively. In order to do

that, people must have adequate training in search techniques appropriate for each database accessed. Most Internet sources offer little or no training in the art of designing and carrying out a high-quality search strategy.

Those considering 'solo' literature searching as their main information-seeking strategy would be well advised to contact their local academic librarian, who should be able to offer help and training in professional search syntax and techniques.

Bernadette Coles

Violet Hughes Library,
Velindre Hospital,
Cardiff CF4 7XL, UK
e-mail: wlbmc@thor.cf.ac.uk

SIR — Ito writes about the necessity to search for both 'sulphide' and 'sulfide' when using a CD-ROM. This problem is worse in some specialities than others. Child health is spelt 'pediatrics' in North America and 'paediatrics' in the United Kingdom, and 'anaesthesia' in Britain is 'anesthesia' in North America. 'Fetus' has almost completely replaced 'foetus', although I see there was a 3:1 split in this spelling in papers in translation from Japan last year; this bears out the experimental observation that a language mutates fastest at its point of origin. All this can make life difficult for a paediatric anaesthetist. For complete information, one has to use the wild card in search algorithms.

Ito's proposed solution to standardize spelling by jiggling a vowel or two would not solve the additional problem that words themselves may be different. I am an anaesthetist in Europe but an anesthesiologist in America, just as orthopods are otherwise orthopedic surgeons or even orthopedists. His solution, in short, would not combat the underlying problem that languages change — which is not a problem but a necessary accompaniment to describe technological innovations and advances that are yet to come.

Oliver Dearlove

Department of Paediatric Anaesthesia,
Royal Manchester Children's Hospital,
Manchester M25 2HA, UK
e-mail: oliver.dearlove@man.ac.uk

Never say never?

SIR — In his News and Views report about superconductors (*Nature* **382**, 760; 1996), David Bishop misattributes to Yogi Berra a quotation by Dan Cook, who said: "The opera ain't over till the fat lady sings". Yogi Berra said: "It ain't over until it's over." Of course, those of us in the academic world know that it's never over.

Michael Katz

March of Dimes Birth
Defects Foundation,
1275 Mamaroneck Avenue,
White Plains, New York 10605, USA
e-mail: katzmi@nyc.pipeline.com

Old fossils could be fractal frauds

Malcolm Walter

Well, are they or aren't they? The debate over whether some of the accretions known as stromatolites are really of biological origin, and thus can provide information about the early history of life on Earth, takes a fresh turn.

STROMATOLITES are the most abundant fossils in rocks representing the first 2–3 billion years of recorded Earth history¹. They reflect the history of bottom-dwelling, mat-forming microorganisms, particularly cyanobacteria but also other bacteria, Archaea and algae—that is, aspects of the history of all three superkingdoms of life. Moreover, they are included in the strategy for exploring for evidence that life once existed on Mars, because they might have formed in lakes and around thermal springs that once occurred there^{2,3}.

But what if they are not fossils at all? On page 423 of this issue⁴, John Grotzinger and Daniel Rothman use the principles of fractal geometry to analyse the morphology of one form of stromatolite from the Proterozoic of Canada. They conclude that the morphogenesis of these stromatolites, and perhaps many others, could be entirely abiotic, or that if 'vital' processes were involved it may be impossible to demonstrate their effects.

Ever since stromatolites were first described in the last century, their interpretation has been contentious. They have been considered to be giant fossils of Foraminifera, sponges, hydrozoans, abiotic concretions, folds and any number of other things. Then in the 1920s and 1930s modern analogues were recognized forming from living mats of cyanobacteria^{5,6}. So the interpretation of the fossil examples as cyanobacterial seemed secure.

This was reinforced when undisputed microbial fossils were found within stromatolites of the Proterozoic Gunflint Iron Formation in Canada⁷, and subsequently within many other examples, and when the large lithified modern stromatolites of Shark Bay in Western Australia were dis-

covered and found to be under construction by communities of cyanobacteria and algae^{8,9} (Fig. 1).

Meanwhile, the interpreters of stromatolites had polarized into two camps. One

Although it has long been recognized, except by the unwary, that abiotic mineral precipitates can mimic features of stromatolites, two relatively recent events have heightened concern about this problem.

The first was the discovery of 3.5-billion-year-old stromatolites, which have assumed prominence as part of the evidence of the earliest life on Earth^{10,11}. But the extent to which they are biogenic is a matter of continuing controversy¹², the magnitude of the problem being illustrated by the fact that there are no Archaeal stromatolites indubitably containing fossils of the microorganisms that built them. The second is the recognition (by Grotzinger) of abundant evidence of precipitation of aragonite and calcite (CaCO₃) on the sea floor before one billion years ago, commonly forming structures previously interpreted as stromatolites¹³. The correct interpretation of these objects can provide important information about the past chemistry of the oceans.

It can be very difficult to demonstrate beyond reasonable doubt that stromatolite-like objects are biogenic, and Grotzinger and Rothman⁴ have tackled the issue in an innovative and rigorous way. Using photographs taken in the field, and polished slabs of rock in the laboratory, they traced and digitized the layers of stromatolites to allow the calculation of mathematical power spectra. They show that, over three orders of magnitude in size, the stromatolite laminae as seen in the field and on the slabs obey the same power law.

From this they obtain the fractal dimension of the stromatolites, and use it to deduce the processes of stromatolite growth.

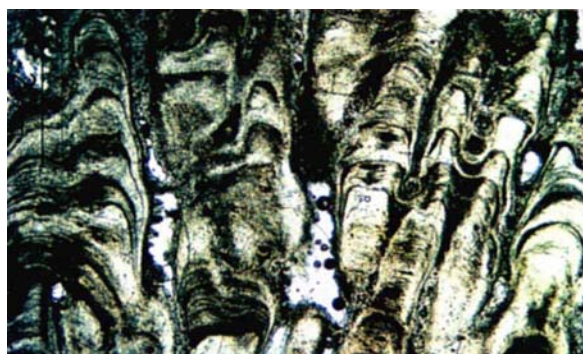
The upshot, they propose, is that the morphogenesis of all stromatolites can be accounted for by four abiotic processes: fallout of suspended sediment, downslope movement of that sediment (or the mathematically equivalent surface-tension effects in chemical precipitation), surface-normal precipitation, and random effects. They conclude that, theoretically, abiotic processes can generate stromatolites, and in the absence of microfossils within

IMAGE
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FIG. 1 Stromatolites at Shark Bay, Western Australia. It was the discovery that these structures are currently being constructed by cyanobacteria and algae that convinced geologists that stromatolites have biological origins.

focused on their biology and use in biostratigraphy (an approach initiated in the 1930s and championed in the Soviet Union but applied widely to this day). The other held the physical aspects of morphogenesis to be dominant, and saw the value of stromatolites as subtle indicators of past environments. This second approach had its strongest advocates in North America. But over the past few decades, polarization has faded: these days, most people working on stromatolites see merit in both the biological and physical approaches.

The main intervals—aeons—of Earth history (Myr, millions of years)		
0 Myr	Phanerozoic	544 Myr
544 Myr	Proterozoic	2,500 Myr
2,500 Myr	Archaean	4,750 Myr



Malcolm Walter

FIG. 2 Thin section of 'spicular geyserite', an opaline silica deposit from the margin of a hot spring in Yellowstone National Park in Wyoming, USA. For a long time, the stromatolite-like geyserite that forms at temperatures of more than 73 °C around hot springs and geysers was thought to be one of the best examples of how physical and chemical processes can mimic those operating in microbial mats to produce stromatolites. Cady and Farmer¹⁸ have, however, shown that microbial biofilms are involved in the construction of geyserite. So although Grotzinger and Rothman⁴ make a good case for the abiogenic formation of stromatolites, life will keep popping into the scene.

them it may be impossible to prove their biological origins.

Nonetheless, Grotzinger and Rothman would probably not disagree with the proposition that there are good reasons for thinking that most stromatolites are biogenic. First, there are examples with microfossils arranged in patterns indicating that they were responsible for the construction of the laminae of the stromatolites¹⁴. Second, Grotzinger himself has shown that the distribution of stromatolite-dominated carbonate sedimentary rocks in the Proterozoic matches that of the algal- and coral-dominated equivalents in the Phanerozoic, reflecting

comparable biological influences¹⁵. Third, there are numerous modern stromatolites where biological influences in their morphogenesis can be directly observed¹⁶.

Finally, and rather ironically in the current context, in the modern world it is difficult to find any stromatolite-like objects that are demonstrably abiotic. For instance, what used to seem like excellent examples of the latter, the high-temperature geyserites of hot springs¹⁷ (Fig. 2), have recently been shown to be inhabited by microbial biofilms that are apparently essential in the morphogenesis of the geyserite¹⁸. The role of biofilms in mineral precipitation and sediment morphogenesis is as yet

little known. But given the pervasive — maybe ubiquitous — presence of life in environments cooler than 150 °C or so,

experience tells us to expect these inconspicuous organic structures to be important players.

However, Grotzinger and Rothman do show that many of the features of stromatolites that are often tacitly assumed to be biogenic are just as likely to be abiotic. It follows from their analysis that we need to take a fresh look at the processes of stromatolite morphogenesis to seek more convincing measures of biogenicity than are currently available. With the techniques that we have at the moment, biogenic and abiogenic stromatolite-like structures may frequently be indistinguishable. Grotzinger and Rothman provide us with a new, rigorous and objective way to approach the interpretation of these structures. This is particularly welcome because, as the burgeoning controversy surrounding the interpretation of the 'microfossils' in the meteorite from Mars reminds us, revealing the geological history of microbial life is not an easy business. □

Malcolm Walter is in the School of Earth Sciences, Macquarie University, New South Wales 2109, Australia.

CONSERVATION BIOLOGY

A-bombs against amphibians

Jared M. Diamond

WITH their permeable skins, shell-less eggs and use of land as well as freshwater habitats, amphibians are sensitive indicators of environmental quality. Hence the concern aroused by worldwide reports of declines in amphibian populations, possibly stemming from the combined effects of many causes. Those causes are very difficult to tease apart, but a new paper by Gamradt and Kats¹ elegantly applies all four methodologies of experimental ecology to establish the nature of the threat posed to one particular amphibian. It turns out that the story is an old one in ecology — predation by introduced species. But the twist in this case is that official policy continues unwittingly to assist the process of decline.

The victim is the California newt *Taricha torosa*, which used to be widespread in coastal California. But when my young son developed a fanatical interest in newts and we began scouring Los Angeles County for the species, we could find none except in a few protected streams. That was surprising, because the newt seems to be a tough customer, with awesome antipredator defences: newt adults and eggs contain tetrodotoxin, the feared nerve poison notorious for its presence in the ovaries of Japanese pufferfish; the eggs are encased in a gelatinous capsule; and the larvae hide when they detect chemical signs of native predators. Might those defences be ineffective

against crayfish (*Procambarus clarkii*) and mosquitofish (*Gambusia affinis*), two predators recently introduced into southern California?

Gamradt and Kats's traverse of methodologies began with so-called natural snapshot experiments², in which one simultaneously compares several sites differing in the presence or absence of a putatively significant ecological factor. Ten streams in Los Angeles County, which were known to have contained newts but not the introduced predators in the 1980s, were re-surveyed in 1994 when one of the streams now contained crayfish, one contained mosquitofish, one contained both predators, and the other seven still contained neither. Lo and behold, the seven streams without the predators also still contained newts, while the first three did not.

Gamradt and Kats went on to a natural trajectory experiment, comparing the same community at different times during a natural perturbation. Heavy rains in the winter of 1994–95 washed crayfish out of Trancas Creek, the stream that had contained crayfish but no newts in 1994 (on the average, 20 crayfish per pool in each of six surveyed pools). In May 1995, the now crayfish-less pools contained nine newts and 24 newt-egg masses per pool. But this year the crayfish recolonized Trancas Creek, reducing the adult newt population by 78% (to two

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per pool) and the egg mass density by 89%, and eliminating newt larvae entirely³.

The laboratory research designed to explore the mechanisms underlying the striking outcome of these natural experiments consisted of placing one newt egg mass or four larvae for one day in each of three tanks — one containing a crayfish, one with a mosquitofish, and one tank with neither. In the absence of predators, newt-egg survival was 100% and larval survival 96%. These numbers declined to 13% and 17%, respectively, in the pres-

ence of crayfish, and 96% and 46% in the presence of mosquitofish. Both predators caught and consumed larvae, and crayfish (but not mosquitofish) could scrape open the egg mass's protective gelatin to expose and consume the embryos.

Finally, to extend their laboratory work to more natural conditions, Gamradt and Kats resorted to a field experiment. Egg masses and larvae were placed in plastic-walled enclosures resting on the bottom of a stream, again in the presence or absence of crayfish or mosquitofish. The results were essentially the same as in the laboratory: egg survival was 95% without predators, and less than 15% with crayfish (mosquitofish were not tested because of their inability in the laboratory to penetrate egg masses); larval survival was 88% without predators, 13% with mosquitofish and zero with crayfish.

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Victim — the California newt *Taricha torosa* — together with one of the villains responsible for its decline, an introduced crayfish; the second villain is another introduced species, the mosquitofish. Inset: a newt tail showing damage from crayfish attacks.

ence of crayfish, and 96% and 46% in the presence of mosquitofish. Both predators caught and consumed larvae, and crayfish (but not mosquitofish) could scrape open the egg mass's protective gelatin to expose and consume the embryos.

Thus, introduced predators suffice to explain the disappearance of California newts in the streams surveyed by Gamradt and Kats. However, the authors do not fall into the error of assuming that the same explanation applies globally. Other factors, including habitat destruction and ultraviolet light, have been implicated in the decline of other amphibian species elsewhere⁵⁻⁹. Indeed, the sensitivity of amphibians to so many factors likely to be

embryos, and can attack adult newts equal to themselves in size (how crayfish withstand the tetrodotoxin, which occasionally kills Japanese sushi-loving humans consuming pufferfish whose ovaries have not been correctly removed, remains a mystery). Larval newts fail to recognize chemical cues from the crayfish and mosquitofish, warning them of danger. That failure is reminiscent of a previous discovery by Kats, Petranksa and Sih⁴: among 15 species of amphibians, the larvae of eight species that regularly encounter native fish in their natural habitats have evolved either to be unpalatable to the fish (presumably because of effective poisons) or to hide when their water contains chemical cues exuded by the fish.

Thus, introduced predators suffice to explain the disappearance of California newts in the streams surveyed by Gamradt and Kats. However, the authors do not fall into the error of assuming that the same explanation applies globally. Other factors, including habitat destruction and ultraviolet light, have been implicated in the decline of other amphibian species elsewhere⁵⁻⁹. Indeed, the sensitivity of amphibians to so many factors likely to be

detrimental to other species (including humans) is what makes their decline so worrisome. If the world's oldest group of terrestrial vertebrates can no longer cope, what prospects do the rest of us newcomers face? First, them; next, us?

Gamradt and Kats's paper contains one statement that I initially could not believe. The authors claim that mosquitofish got into the streams of Los Angeles County in the first place through being distributed freely by county government mosquito-control personnel (to eat mosquito larvae). Incredulous, I phoned the Los Angeles County Mosquito Abatement District at 310-915-7370. In answer to my questions, a staff member told me: "Yes, they would give me mosquitofish; no, there would be no cost to me; no, I would not have to identify myself, fill out an application or explain what I intended to do with the fish; no, the fish are harmless and present no dangers of which I should be aware; yes, I could have 100 of them".

To set this information in perspective, reflect that California has suffered economic losses of billions of dollars in recent years, and that state agencies are spending uncounted millions of dollars annually on control programmes because of other introduced species that have proved disastrous for agriculture or the environment. These species include the notorious Mediterranean fruitfly, abalone fan worm, African bee, cabbage butterfly, garden snail and ice plant. As I reflected on this recent history, I realized that the 100 mosquitofish that I had been offered by a different government agency might suffice, if released at well-chosen sites, to exterminate all newt populations still surviving within the 4,000 square miles of Los Angeles County. In that light, my phone call with the county staff member made me feel as if I were a resident of a heavily bombed city who calls a local government office and is told "Yes, you may have some A-bombs to excavate your construction site", and "No, the bombs present no hazards and are being distributed gratis". □

Jared M. Diamond is in the Department of Physiology, University of California Medical School, Los Angeles, California 90024-1751, USA.

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Stirrings of the Galactic heart

Mark Morris

WHAT lies at the very centre of the Galaxy — that point about which the entire Milky Way turns? One way to answer the question is to look at the movements of stars in its vicinity. The first direct measurements of the proper motion of stars near the Galactic Centre are reported by Eckart and Genzel on page 415 of this issue¹. When combined with the best and most recent radial velocity data², they show with little ambiguity that about 2.5 million solar masses of dark matter lurks within a relatively tiny region at the centre.

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The centre of the Galaxy. The motions of stars in the central light year of this 80×80 light-year infrared image show that 2.5 million solar masses is concentrated within about 0.1 light yr of the Galaxy's dynamical centre, probably in a huge black hole that also produces the spot of radio emission shown in the inset¹⁰ (2 light yr across).

If a cluster of stars is bound (that is, if they aren't flying apart) and in equilibrium, the spread of stellar velocities is determined by the total mass of the cluster, just as the orbital velocity of the Earth is determined by the mass of the Sun. But until now, our knowledge of the dynamics of stars near the centre of our Galaxy has been limited to observations of a single velocity component — the radial, or line-of-sight, velocity, as measured by its Doppler effect on atomic spectral lines. The application of Newton's law of gravitation to draw conclusions about the distribution of mass in the Galactic Centre has therefore always required some assumption about the missing velocity components.

For some time, radial-velocity measurements have nonetheless yielded tantalizing evidence for the presence of a compact, central concentration of non-luminous matter weighing in at a few million times the mass of the Sun, but the evidence has always been accompanied by a nagging uncertainty because the necessary assumptions about the unobserved

velocity components had never been proved valid. Indeed, it was possible to choose assumptions (albeit rather extreme ones, with most of the stars on highly elongated orbits) that considerably reduced or even eliminated the need for non-luminous matter.

Those missing velocity components — the projections of stellar space velocities onto the plane of the sky — can be seen in nearby stars as 'proper motion' against a distant backdrop. The 25,000-light-year distance to the Galactic Centre places it well beyond the usual realm of proper-motion measurements, but fortunately the distance is partly compensated for by the large stellar velocities in the central light year of the Galaxy (a few hundred km s^{-1}). Shift-and-add imaging and image deconvolution techniques are employed by Eckart and Genzel to counteract the blurring effect of the Earth's atmosphere, and give them the high spatial resolution they need to obtain significant measurements within their five-year time base.

The main result of these measurements is that, at all projected distances from the Galactic Centre, the average absolute velocity of stars along any axis in the plane of the sky is the same as that along the line of sight. That is, the stellar velocity dispersion is everywhere isotropic. Past analyses have often assumed isotropy, but now that it is established, the velocity dispersions can be translated into an enclosed mass with some reliability. Those early, naively derived masses are correct, and the conclusion that there is a sizeable dark mass at the Galactic Centre now rests on firm ground. The inferred mass cannot be in the form of normal stars, because the light they would emit could easily be seen at near-infrared wavelengths.

What makes this conclusion even more poignant is that the dark mass happens to be confined to within about a tenth of a light year of a unique object: the bright, unresolved radio source called Sagittarius A* (ref. 3). This source fulfils the expectations of many theorists as to how a disk of hot gas accreting onto a black hole should manifest itself to observers^{4,5}. The measurements of Eckart and Genzel are

consistent with the possibility that all the inferred dark mass is in Sgr A*. If so, its few million solar masses probably make it by far the largest black hole in the Galaxy, though still a lightweight compared with the compact objects of 10^7 to 10^9 solar masses or more, inferred from the dynamics in the nuclei of some other galaxies⁶.

Even if Sgr A* is a black hole, its mass is not yet well determined. It might be substantially less than a few million solar masses⁷, in which case there would be room for other forms of non-luminous matter. One of the most likely candidates is a population of stellar-mass black holes. The central stellar cluster contains dozens of massive, luminous stars, which, if they are normal stars and not exotic objects, are doomed to very short lifetimes before they explode as supernovae, leaving behind compact remnants such as neutron stars or black holes, each of 1 to 20 solar masses. Over the ten-billion-year lifetime of the Galaxy, it is possible to build up enough remnants at the centre to account for some fraction of the non-luminous matter there^{8,9}.

But the size constraint placed by the new observations on a cluster of stellar remnants is severe. Any such cluster must have undergone a core collapse if it is to fit into the tiny volume allowed. This constraint will be pushed even further in the near future, when even higher-resolution observations, currently underway, are brought to bear on the proper motions of the innermost portions of the central stellar cluster.

Eckart and Genzel give us a taste of what is to come with their preliminary observations of the Sgr A*(IR) cluster, a group of infrared sources — presumably stars — tightly clustered about Sgr A*. If the proper motions of these relatively dim objects are as large as the data are beginning to imply, it will be difficult to avoid the conclusion that Sgr A* has all the dark mass. The question would then be, what happened to all the stellar remnants? □

Mark Morris is in the Department of Physics and Astronomy, University of California at Los Angeles, Los Angeles, California 90095-1562, USA.

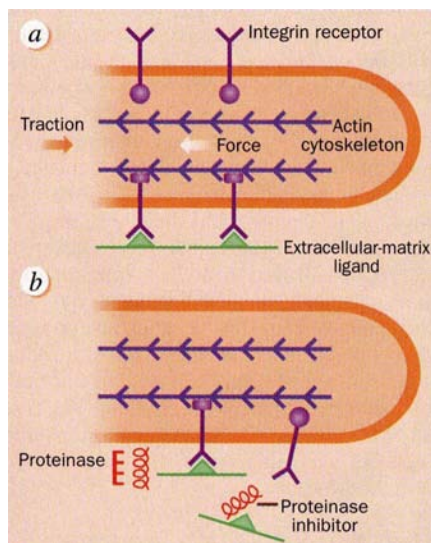
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Making connections count

Douglas A. Lauffenburger

CELLS in motion have been examined by biologists from as many angles as the proverbial elephant by the blind men, yet we still know very little about the basic organizing principles of cell motility. But reports on pages 438 and 441 of this issue^{1,2} provide significant new information, mainly concerning basic aspects of the physical linkages between key molecular components involved in force transmission.

Force generated intracellularly by motor proteins associated with the actin cytoskeleton must be transmitted to ligands anchored on the substratum. Transmission typically occurs through transmembrane receptors, known as integrins, which are specific for extracellular-matrix ligands. To allow cell movement, this transmission must be accomplished in a manner that permits linkages at the front of the cell to provide stable traction for the applied stress, at the same time as those at the back of the cell are unstable. Hence, the molecular processes governing these linkages are a major issue in research.



a, Summary of the conclusions of Felsenfeld *et al.*¹. Binding of an extracellular-matrix ligand to the integrin receptor (possibly associated with receptor aggregation) induces linkage of the receptor to the actin cytoskeleton. Molecular motors generate a force on the cytoskeleton and the linked receptors, so that receptors linking the cytoskeleton to the substratum generate traction which could pull the cell body forward (depending on linkages at the back of the cell). b, Summary of the conclusions of Stefansson and Lawrence². Proteinase inhibitor can block the binding of the extracellular matrix ligand to the integrin receptor, thereby preventing linkage of the receptor to the actin cytoskeleton for force transmission. As well as enzymatically degrading the matrix, proteinase can bind to the inhibitor and release the block to receptor/ligand binding. Accessory molecules mediating the linkage between the integrins and the cytoskeleton are not shown.

Felsenfeld *et al.*¹ show that binding of integrins by a ligand in the extracellular matrix engages the cytoskeletal clutch, allowing transmission of intracellular motor forces to extracellular substrata (a in the figure). And although blocking of receptor/ligand binding might be an obvious way to reduce traction, Stefansson and Lawrence² discover a surprising source for a blocking agent — a proteolytic enzyme inhibitor (b in the figure).

Felsenfeld *et al.* use videomicroscopy to follow (on the dorsal surfaces of fibroblasts) the trajectories of 40-nm colloidal gold particles coated with the integrin-binding domain of the extracellular matrix protein fibronectin. After binding, the gold particles diffuse randomly on the cell surface for less than a second, before undergoing sustained linear transport towards the rear of the cell. Earlier work indicated that this sort of directed membrane transport is mediated by the cytoskeleton³, and involves biochemically specific properties of the cytoplasmic domain of a cell-surface receptor⁴. It is generally assumed that the same intracellular motile force that can pull a mobile bead backwards can pull cellular components forward when linked to a relatively immobile extracellular substratum⁵. So the binding of an integrin by its ligand leads quickly to the force-transmission linkage required for cell locomotion.

Felsenfeld *et al.* also found that this linkage was rapid and dynamic. Integrins 'passively' tagged with colloidal gold particles coated with an anti-integrin antibody (not competing for the ligand binding site) alternated abruptly between diffusive and directed trajectories when a soluble integrin-binding peptide ligand was added. Presumably, directed transport occurs while the ligand occupies the receptor, whereas diffusive transport occurs when the receptor is unoccupied.

Apparently, the cytoskeletal force causing the directed transport of ligand-bound integrins is activated by another stimulus. This is independent of integrin ligand binding, as 1- μ m polystyrene beads coated with the noncompetitive anti-integrin antibody are also transported towards the back of the cell⁴. In this case, the receptor/cytoskeleton connection seems to be elicited by a certain degree of integrin aggregation, because the much smaller 40-nm colloidal gold particles are not pulled backwards when coated by this antibody (except when soluble integrin-binding ligand is added, as mentioned above)⁴. This finding can be compared with the results of work on cytoskeletal association in non-migrating cells with focal adhesions (strong anchorage points between the plasma membrane and the

substratum)⁶. In that situation, both integrin ligand binding and aggregation, as well as downstream kinase activation, are required for linkage to actin filaments. Aggregation could also be occurring in the experiments of Felsenfeld and colleagues, as they point out. Nonetheless, it seems that integrin organization in migrating cells is not the same as that in stationary cells with focal adhesions⁷.

If integrins can be linked to a force-generating cytoskeleton by ligand binding, blocking that binding could provide a means for external control of cell locomotion (b in the figure). In the second paper, Stefansson and Lawrence² identify a physiological blocking factor — plasminogen activator inhibitor-1 (PAI-1) — that diminishes the ability of smooth-muscle cells to migrate on vitronectin-coated substrata. Urokinase plasminogen activator (UPA) is believed to promote cell migration in wound healing *in vivo*, by locally breaking down extracellular-matrix fibrils: PAI-1 inhibits this matrix proteolysis⁸. Although they take nothing away from that function, Stefansson and Lawrence show that PAI-1 can reduce vitronectin receptor-mediated migration *in vitro*, by binding directly to vitronectin itself and blocking its ability to interact with the vitronectin receptor. Moreover, they show that this effect of PAI-1 is independent of its inhibitory effect on the UPA enzymatic activity.

Thus, PAI-1 can inhibit smooth-muscle cell migration by a biophysical mechanism, weakening the adhesion-based traction provided by binding of the integrin to the matrix ligand. The authors² reasonably speculate that proteinases such as UPA may at least partially promote cell migration through extracellular matrices by selectively releasing the adhesion interference caused by their associated inhibitors, along with general destruction of matrix. The story is likely to become more complicated yet, involving the cell receptor for UPA as well⁹. But these observations suggest that biophysical effects are at play in adhesive interactions between the cell and the substratum.

Adhesion-based mechanisms for modulating cell locomotion are especially intriguing because they may not only

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provide controlled and reversible effects, but also either inhibition or enhancement of movement. Data are accumulating in support of a biphasic influence of cell/substratum adhesiveness on migration speed¹⁰. So, depending on the level of cell-surface integrin and corresponding extracellular-matrix ligand, interactions between proteinase/inhibitor pairs such as UPA/PAI-1 could alter the cell/substratum adhesiveness, either towards or away from that yielding maximal migration. Experimental evidence consistent with this bidirectional control has been found in a simpler model system involving a soluble integrin-binding competitor¹¹.

Both of these papers^{1,2} illustrate the central importance of the physical processes

underlying cell locomotion^{12,13}, and show how molecular interactions can influence the linkages between the matrix, the receptor and the cytoskeletal motor. Ground for future explorations will thus be found in understanding how these linkages are regulated biochemically, for instance by growth-factor signalling, and especially how this regulation might provide physical asymmetries in force transmission across the length of the cell to permit forward movement of the cell body^{5,12}. □

Douglas A. Lauffenburger is in the Department of Chemical Engineering and Center for Biomedical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA.

SOLID-STATE PHYSICS

Revolution in magneto-optics

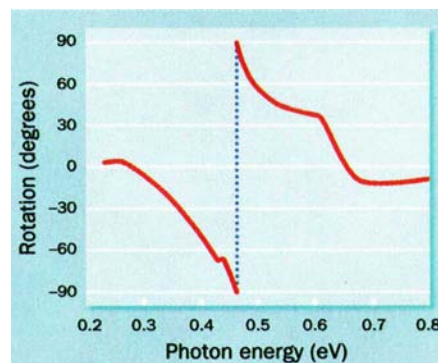
J. A. C. Bland

WHEN light is reflected from the surface of a ferromagnet, its plane of polarization rotates. This magneto-optical Kerr rotation has been with us for a long time, having first been discovered in the late nineteenth century¹, but so far the size of the rotation we can produce remains disappointingly small — typically a few degrees, which is much the same as with the polished magnet pole-pieces of Kerr's original experiment. As a result, only a small optical signal can be generated by reversing the material's magnetization with an external field. (Even so, the advent of the laser and advanced signal detection methods has made it possible to detect even tiny Kerr rotations, and the effect is already used in rare-earth/transition-metal alloy films for erasable information storage.) But now a remarkable 'giant' Kerr rotation through 90° has been observed by Pittini *et al.*² in a single cerium-antimony crystal. The peak effect occurs for incident infrared light with an energy of 0.46 electron volts.

Not only is this the first time that such a large Kerr effect has been seen (the previous record being 14°, in the same material³), but no theoretical prediction of such a large effect has been made, despite several investigations of cerium- and antimony-based compounds⁴. By definition, the largest Kerr rotation possible is 90°, as it is the oscillating electric field vector that is rotated and so +90° and -90° are equivalent. This rotation is so large that it renders invalid the usual theoretical approximations, which relate the polarization change to the off-diagonal terms in a material's conductivity matrix (those that represent currents induced perpendicular to an applied electric field).

From the point of view of applications, big is good, so of course giant would be better. Much research effort has been concen-

trated on developing magnetic multilayer materials, such as those based on ultrathin cobalt-platinum layers (a few ångströms thick) which show sizeable Kerr rotations at the ultraviolet wavelengths needed for high-density recording applications. (A laser reads the rotation produced by each



The giant rotation of polarized light on reflection from a cerium-antimony crystal (from ref. 2).

magnetic domain, and so the resolution is determined by the laser wavelength.)

Other magnetic properties are required for magneto-optical recording applications: in 'perpendicular recording', there must be an internal magnetic field (the 'anisotropy field') favouring a magnetization perpendicular to the magnetic film plane; and in thermomagnetic recording, the Curie temperature (above which the magnetization is lost) must be tuneable, so as to allow 'writing' by a laser pulse, and the stability of the magnetization to external fields must be large enough at room temperature to prevent the medium from being demagnetized.

The finding of Pittini and co-workers is unlikely to revolutionize magneto-optical recording, however, requiring as it does a single crystal, high fields (five tesla) and

low temperatures (1.5 K). Moreover, the 0.46-eV energy of the peak magneto-optical rotation requires a reading wavelength of more than a micrometre — too long to be of use in high-density recording. In general, the size of the rotation is reduced in polycrystalline materials compared with single crystals, so thin films of CeSb will certainly show a smaller effect. Nevertheless, it is conceivable that a niche application could be found for CeSb, such as a field-controllable optical isolator or switch, which requires such a large optical rotation.

There was already a hint that a large rotation existed in CeSb: in 1986, Reim *et al.*³ saw that the Kerr effect increased as the photon energy was reduced. It still rose as the energy was reduced to 0.5 eV, and showed no sign of a maximum, but with their apparatus they couldn't reach the all-important energy region.

In part, the giant effect in CeSb can be explained as a fortunate concurrence of several effects. At 0.46 eV there is a 'plasma minimum': the incoming radiation excites a collective motion of the electrons, which screens the electrical polarization inside the material. That reduces those components of the reflectivity that do not change the polarization of incident radiation, so a large rotation is usually seen near a plasma minimum⁵. Microscopically, the Kerr effect arises from spin-dependent transitions between initial and final electron states. The lower level involved in the magneto-optical transition in CeSb is a 'saturated state', that is, its component of angular momentum parallel to an external field has the most negative value possible, and so the state can only absorb right-handed circularly polarized light (which increases the angular momentum).

But these factors can only explain part of the rotation. If we are to explain the giant effect fully, we must understand the details of the transition much better than we do: in particular, the precise nature of the 4f state in CeSb needs to be described better. The key issue is how it differs from the same state in the isolated cerium atom, owing to electronic interactions.

The 90° rotation found by Pittini *et al.* challenges our understanding of these rare-earth compounds, and new theoretical efforts are needed to describe the magneto-optics of CeSb and related materials. Such an effort may in turn play an important part in the search for other magneto-optical materials suitable for future applications. □

J. A. C. Bland is at the Cavendish Laboratory, University of Cambridge, Cambridge CB3 0HE, UK.

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Help from fast synapses

Erwin Neher

PRESYNAPTIC terminals have resisted biophysical analysis for decades. We have known since the classical experiments by Katz and associates¹ that calcium ions are the immediate trigger for release of neurotransmitters. But, because of the inaccessibility of the tiny structures involved, details of the kinetics and magnitude of this signal have remained a mystery.

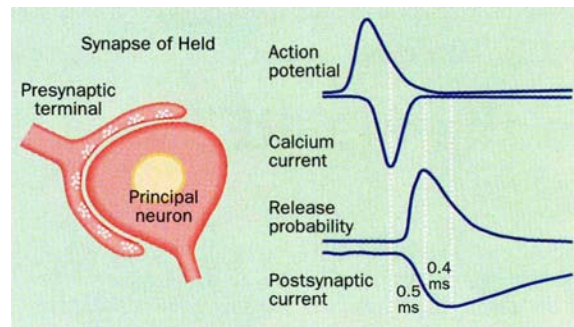
On page 431 of this issue², in a remarkable example of experimental skill and precision, Borst and Sakmann describe some of the signalling features of a mammalian synapse. They resolve with unprecedented accuracy the time course and size of pre- and postsynaptic currents, and address the question of how many calcium channels need to open in order to release the contents of a vesicle into the synaptic cleft.

Most synaptic terminals in most animal species are very small (rarely more than 2–5 μm in width). There are exceptions, however, and notably they are found at synapses that are specialized for fast, precisely timed transmission. The prime example is the squid giant synapse, where a finger-like presynaptic process, several hundred micrometres in diameter, transmits the signal to the giant axon, eventually leading to contraction of the squid's mantle during the escape response. A large-diameter axon enables rapid propagation of an action potential. In turn, the low impedance of such a large structure requires a large synapse, so that the spike is triggered rapidly.

For many years, the squid giant synapse has been the only system in which the presynaptic voltage signal could be clamped while simultaneously recording postsynaptic responses^{3,4}. But there are also structures within the vertebrate central nervous system where relatively large synapses have evolved — probably owing to a similar requirement for high-speed transduction. For instance, bipolar nerve terminals from goldfish retina are large enough to perform 'whole-terminal' patch-clamp recordings^{5,6}; likewise, the ciliary ganglion and the nucleus magnocellularis of chicken contain calyx-like terminals that allow stable presynaptic recordings^{7–9}. No one, however, has yet managed to carry out high-speed and simultaneous voltage-clamp recording from both the pre- and postsynaptic sides in any of these preparations.

Borst and Sakmann's achievement² is to have done this, employing the so-called synapse of Held in the brainstem of rats. Forsythe¹⁰ first showed that this structure could be patch clamped, work that was

taken further last year by Borst, Helmchen and Sakmann¹¹ who explored the conditions under which clamping could reliably be carried out. Now, Borst and Sakmann have determined the precise timing of the individual steps in neurotransmission: the up- and downstroke of the presynaptic action potential; the rate and magnitude of calcium influx and subsequent transmitter release; and the overall postsynaptic response.



Borst and Sakmann² have looked at the precise timing of events in the calyx synapse of Held from rat brainstem. The process involves about 100 or more active zones (sites of vesicle accumulation), each of which releases one to two vesicles of neurotransmitter in response to an action potential. During the action potential, a total of 0.9 picocoulombs of charge carried by calcium ions enters the presynaptic terminal, constituting a wave of calcium entry that lasts for 0.35 milliseconds during the downstroke (recovery phase) of the presynaptic action potential. Neurotransmitter release is detected on average 0.5 ms later, leading to an excitatory postsynaptic current that peaks 0.9 ms after the peak of calcium entry into the presynapse.

They find that calcium influx is tightly associated with the repolarizing phase of the action potential, and has a 'half-width' of about 350 μs . The ensuing release events can be detected at the postsynaptic membrane under low external concentrations of calcium as miniature synaptic currents with a mean latency (the delay from the peak of calcium influx to the beginning of a miniature current) of only 500 μs . Full-size postsynaptic currents, as recorded with normal concentrations of extracellular calcium, can be reconstructed by superimposing the waveforms of miniature currents according to their latency distribution. This confirms a finding by Isaacson and Walmsley¹², and shows that release probability has the same time course at high (2 mM) and low (0.25 mM) concentrations of external calcium, although the total extent of release changes by two orders of magnitude over this range.

That the rate and magnitude of neurotransmitter release are independent of each other, and of the concentration of extracellular calcium, has puzzled bio-

physicists for decades. Many scientists believe that the rate of release is governed by a rapid build-up and then collapse of very restricted 'domains' — areas of increased calcium around open channels^{3,13,14}. Borst and Sakmann concentrate on this aspect, and ask whether a domain resulting from the opening of a single channel is enough to trigger the release of a vesicle of neurotransmitter, or whether several overlapping domains from many channels are required.

To this end, they first compared the number of calcium channels opening at the synapse during an action potential with the number of vesicles released. They conclude that at least 60 channels open per release event. Second, they studied the effect on synaptic strength of adding calcium chelators to the presynaptic intracellular saline. This approach has been taken before in studies of other forms of calcium signalling, and has the aim of intercepting calcium ions before they bind to their natural targets. The expectation is that the closer the target is to the calcium source, the less likely it is that the chelator will interfere with the phenomenon under study.

Surprisingly, Borst and Sakmann find that even a 'slow' calcium chelator — such as EGTA — is able to clear out calcium at concentrations as low as 1 mM, as judged by the reduction in neurotransmitter release. When compared to the earlier result¹¹, that a 'fast' chelator (BAPTA) is effective at similar concentrations, this finding takes on an extra dimension. It shows that kinetic competition

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between the chelator and the natural target cannot be the full explanation for the observed effects. If it were, BAPTA would be more than 100 times more effective than EGTA, because its calcium-binding rate constant is that much higher. Such a large difference in calcium inhibition has indeed been found at the squid giant synapse¹³, but at the synapse of Held the difference is only a factor of four to ten.

So why does BAPTA not live up to the expectations stemming from its high calcium-binding rate? A likely reason is that its calcium-free form is locally depleted while channels are open. But — crucially — this cannot happen in a single-channel domain. The equations of Pape *et al.*¹⁵, applied to the case under consideration,

show that it takes 1.9 picoamperes of calcium current to deplete 1 mM BAPTA by a mere 20 per cent. This is beyond any estimate for a single-channel calcium current. So the results can be taken as further evidence that many channels have to cooperate to produce the neurotransmitter release.

On the other hand, the implication that regions of elevated calcium may be rather extensive could conflict with the finding that the rate of neurotransmitter release is independent of the extracellular concentration of calcium; this is because extensive depletion of free buffer molecules over larger areas would slow down the time course of calcium decay following calcium inflow. It will be interesting to see

whether numerical simulations of the diffusional spread of the calcium signal can provide a set of parameters that allow for both the weak action of BAPTA (relative to EGTA) and the invariance of the rate of release. If so, an answer regarding the importance of 'residual calcium' or 'residual bound calcium'²¹⁴ for short-term synaptic plasticity may be at hand. It will be even more interesting to look at the action of reagents, other than calcium chelators, that interfere with specific molecules at the release site. □

Erwin Neher is in the Abteilung Membranbiophysik, Max-Planck-Institut für Biophysikalische Chemie, Am Fassberg, D-37077 Göttingen, Germany.

GAMMA-RAY ASTRONOMY

A keener eye than EGRET

Peter J. T. Leonard

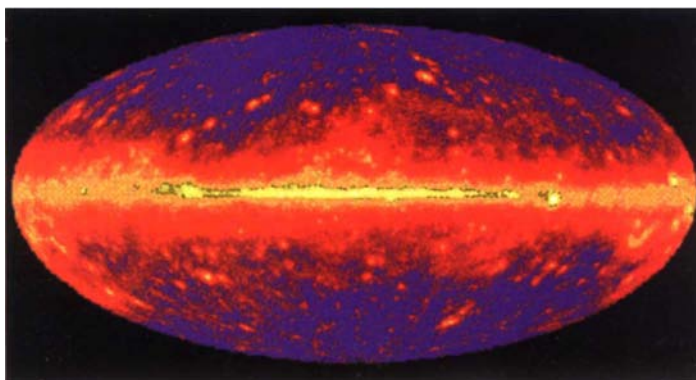
In these fiscally tight times, NASA has asked astronomers only to propose new missions that are at least an order of magnitude better than previous satellites. The high-energy γ -ray response to this challenge is called GLAST, the Gamma-ray Large Area Space Telescope, the scientific case for which was presented at a meeting last month*.

The best high-energy γ -ray instrument to date is EGRET (Energetic Gamma-Ray Experiment Telescope) on board the Compton gamma-ray observatory. GLAST will be roughly one to two orders of magnitude better than EGRET in terms of detectable photon energy, field of view, sensitivity and angular resolution (see table opposite). It promises to rewrite the book on the phenomena observed by EGRET, and to discover new ones, while costing less (under \$300 million).

Our understanding of every known high-energy phenomenon in the Universe should be improved by GLAST. Three examples are blazars, young pulsars and γ -ray bursts.

Blazars are luminous and rapidly variable active galactic nuclei (a term encompassing any brilliant source associated with galactic centres, including quasars and radio galaxies). EGRET discovered that blazars are strong γ -ray sources — in fact, the luminosity of the galaxy 3C279 is dominated by γ -rays. Relativistic beaming

is necessary to explain the variability and power of blazars, and so we must be viewing these objects along a jet gushing from the central black hole (M. Urry, Space Telescope Science Inst., Baltimore, MD). So γ -ray observations of blazars may pro-



The simulated γ -ray sky, as seen by the proposed satellite GLAST. Cosmic rays interact with interstellar gas in our Galaxy to produce the diffuse γ -ray Galactic background. If cosmic rays come from supernova remnants, as many theorize, then GLAST should be able to see enhanced γ -ray emission from cold, dense gas clouds that just happen to lie near them. The point sources scattered across the sky are blazars.

vide us with our best opportunity to study the origin of astrophysical jets, and what happens close to a supermassive black hole. GLAST would provide us with a few hundred blazars to study in detail, far more than the handful seen by EGRET.

Some blazars produce huge γ -ray outbursts (J. Mattox, Boston Univ., MA) in which the emission can increase by an order of magnitude. Multi-wavelength observations show that these γ -ray flares appear to be related to the birth of radio-emitting regions in the jets. Forty times more sensitive than EGRET to such events, GLAST should produce much more detailed light curves and spectra.

EGRET has also detected seven γ -ray pulsars; GLAST should see hundreds. Observations of γ -rays in the GeV range may be the best way to locate and study young pulsars, because they typically lie in regions of high electron density where their radio emission can be greatly attenuated (R. Romani, Stanford Univ.). Similarly, young pulsars with massive binary companions that have strong winds are also more likely to be detected at γ -ray than

radio wavelengths. GLAST should even be able to tell whether the pulsed emission is produced at the pulsar's polar cap or by a process occurring near a gap in the magnetosphere (A. Harding, Goddard Space Flight Center, Greenbelt, MD).

Perhaps the most baffling mystery in all of astrophysics is what produces γ -ray bursts. The temporal diversity of these brief flashes of radiation makes them hard to explain, and it is still unclear whether they are at cosmological or galactic distances. GLAST has a far smaller dead time than EGRET, and that would permit a more accurate assessment of the

high-energy γ -ray contribution to the bursts — especially if it comes in pulses, as it does in the MeV range. In addition, the discovery of a spectral turnover at high energies would imply a cosmological origin. The most promising way to solve the γ -ray burst mystery, however, remains to detect a counterpart in the sky at longer wavelengths, and GLAST's higher angular resolution should narrow the search to a much smaller area.

So how is it to be built? There are three promising technological paths for the particle trackers in GLAST: silicon strips, gas microstrips and scintillating fibres. All three involve the same basic

*Workshop on the Next Generation High-Energy Gamma-Ray Telescope, Greenbelt, Maryland, USA, 4–6 September 1996.

Comparison of EGRET and GLAST parameters

	EGRET	GLAST design
Energy range	30 MeV to 30 GeV	10 MeV to 300 GeV
Field of view	0.62 steradians	5.6 steradians
Point source sensitivity above 1 GeV	1.2×10^{-8} photons cm^{-2} steradian $^{-1}$	1.5×10^{-10} photons cm^{-2} steradian $^{-1}$
Point source location	5–10 arc minutes	0.5–1 arc minutes

physics: an incident γ -ray strikes a heavy metal 'converter material' to produce an electron-positron pair. They are tracked to reveal the γ -ray's direction, and its energy is measured by a calorimeter behind the tracking system. Cosmic rays would produce a spurious signal, but they can be identified by a plastic anti-coincidence shield around the detector.

In principle, GLAST refers to whatever the next large-area γ -ray satellite turns out to be, but the name is usually associated with a design based on silicon strips. Each tracking tower in a 7×7 array would have a detection area of $24 \times 24 \text{ cm}^2$, and a dozen silicon strips placed over its height. This is the design referred to in the table; see <http://www-glast.stanford.edu> for details. The calorimeter would be a caesium-iodide crystal scintillator. This version of GLAST could double as a cosmic-ray detector, and test theoretical predictions about the cosmic-ray electron spectrum.

An instrument based instead on gas microstrip technology could in principle provide a very large collecting area and high angular resolution. Anodes and cath-

odes are etched in Pyrex, and immersed in an argon-xenon gas mixture that is ionized by the electrons and positrons (K. Pitts, Univ. Louisville, KY). This technology is still maturing — especially the vital gas purification and recycling system needed to avoid gain degradation — and is well worth further development for uses in medical imaging, such as

mammography.

SIFTER (ScIntillating Fiber Telescope for Energetic Radiation) as described by Geoffrey Pendleton (Univ. Alabama) is perhaps the most underrated technology of the three. It consists of many grids of glass fibres sandwiched between layers of a converter material such as lead. The electron-positron pairs are tracked from the flashes they produce in the fibres. A unique feature of this approach is that the glass fibres can also act as a calorimeter, simplifying the detector. Astronomers hope to launch a 1.1×1.1 -m prototype in a balloon some time in the next couple of years.

History is quite clear; better satellites invariably lead to scientific discoveries previously not even dreamed of. If we seize the opportunity to launch GLAST early in the next century, the best years of discovery in γ -ray astronomy are likely to lie just around the corner. □

Peter J. T. Leonard is in the Compton Gamma-Ray Observatory Science Support Center, Goddard Space Flight Center, Greenbelt, Maryland 20771, USA.

NEUROBIOLOGY

Unwrapping myelination

Greg Lemke

HIGHER-vertebrate nervous systems face a daunting design challenge — the need to pack a relatively large number of nerve fibres, or axons, into a relatively small space (for example, our skulls). The answer is myelin, an insulating sheath that greatly accelerates the rate at which nerve impulses are propagated. Without this sheath, vertebrate nervous systems are largely non-functional, and human diseases that seriously compromise the integrity of the sheath, such as multiple sclerosis, are invariably debilitating.

Myelin is elaborated as an organelle of oligodendrocytes and Schwann cells, in the central and peripheral nervous systems respectively, and consists of an enormous sheet of plasma membrane that is repeatedly wrapped and then very tightly compacted around axons¹. An important mediator of the myelin compaction

process in the peripheral nervous system is protein zero (P_0), an integral membrane glycoprotein that accounts for most of the protein in myelinating Schwann cells². Writing in *Neuron*³, Shapiro and colleagues now announce an X-ray crystal structure, to a resolution of $1.9 \text{ \AA}$, for the extracellular domain of P_0 ($P_0\text{ex}$). This structure turns out to have implications for the mechanics of myelin membrane assembly, the thermodynamics of interaction for immunoglobulin-related adhesion proteins and, perhaps most importantly, the molecular genetics of inherited neurological diseases⁴.

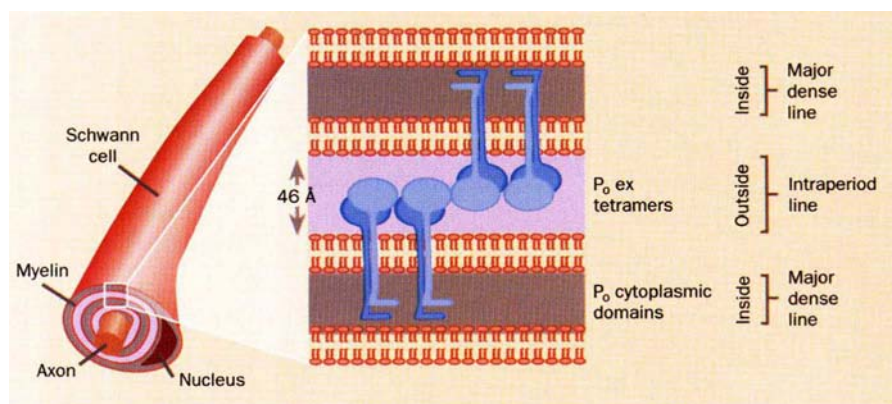
The spiral wrapping and compaction of myelin membrane around axons generates two distinct membrane appositions at each turn of the sheath — one between apposed extracellular membrane faces (the myelin 'intraparallel line') and another

between apposed cytoplasmic membrane faces (the 'major dense line')¹. The two surface appositions formed during the rolling up of a sleeping bag are a good macroscopic analogy. $P_0\text{ex}$ is found at apposed extracellular membrane faces of the sheath, and a variety of experiments have shown that it is essential for membrane compaction at the intraperiod line. The P_0 cytoplasmic domain is similarly implicated in myelin compaction at the major dense line.

$P_0\text{ex}$ has an interesting amino-acid sequence, similar to that of a single variable region of immunoglobulin heavy chains⁵. This feature is shared by a number of cell-adhesion proteins that are involved in homotypic binding interactions. Not surprisingly, then, $P_0\text{ex}$ is homophilic, and seems to 'zip up' the intraperiod line in myelin. The process involves a reaction in which a $P_0\text{ex}$ molecule that is tethered by its transmembrane domain to one extracellular membrane face binds homotypically to another $P_0\text{ex}$ tethered to the apposed extracellular membrane face^{6,7}. Mice that lack P_0 manifest a severe dysmyelinating nerve disease that is characterized by a nearly complete failure in intraperiod-line compaction in the peripheral nervous system⁸.

Knowing the $P_0\text{ex}$ structure at atomic resolution is particularly valuable because a large number of mutations in the human P_0 gene — many of them missense mutations — have already been identified. These mutations result in inherited peripheral neuropathies that range from mild (Charcot-Marie-Tooth type 1B disease, CMT1B) to severe (Déjérine-Sottas syndrome, DSS)^{4,9}. This means that we are in the unusual position of already having the information required to carry out detailed structure-function and genotype-phenotype comparisons. So, what does the $P_0\text{ex}$ structure actually look like? And can one make sense of the variable phenotypes of peripheral-nerve diseases that are associated with $P_0\text{ex}$ mutations in light of this structure?

In line with earlier predictions, the $P_0\text{ex}$ crystal of Shapiro *et al.*³ is made up of a series of ten antiparallel β -strands arranged into two facing β -sheets, just like all variable heavy-chain immunoglobulin domains. But $P_0\text{ex}$ crystallizes as a tetramer rather than as a monomer, and it is likely that such a tetramer is assembled on top of each bilayer at the myelin intraperiod line. A single $P_0\text{ex}$ tetramer looks rather like a square-shaped doughnut with a large central hole, and is thought to interact in a side-by-side fashion with the outside surfaces of four other tetrameric doughnuts tethered to the apposed membrane surface. (All of the monomers in a given P_0 tetramer are linked through their transmembrane domains to the same myelin membrane



Rapidly conducting axons in vertebrate nervous systems are ensheathed by myelin, which is a sheet of plasma membrane wrapped many (up to 100) times around an axon (left). The sheet is tightly compacted, so that in cross-section it resembles a spiral of stacked plasma-membrane bilayers. Protein zero (P_0), an immunoglobulin-related adhesion protein specific to Schwann cells, is an essential mediator of membrane compaction in peripheral nervous system myelin (right). The P_0 extracellular domain (P_0 ex), the X-ray crystal structure of which has now been solved by Shapiro *et al.*³, mediates membrane adhesion at apposed extracellular-membrane surfaces to form the myelin intraperiod line, while the P_0 cytoplasmic domain links apposed cytoplasmic membrane surfaces to form the myelin major dense line.

bilayer.) In this way, a large, planar lattice of interacting P_0 tetramers on apposed membranes may be assembled³. The vertical distance between apposed extracellular membrane faces predicted by this lattice — 46 Å — agrees precisely with measurements of this distance made through X-ray diffraction of intact myelin.

Both the position and genetics of many of the known mutations in P_0 ex make sense in light of the crystal structure^{3,4}. For example, CMT1B-associated missense mutations at Asp 61→Glu, Lys 67→Glu and Arg 69→Ser or His all cluster near the interface between individual monomers in the tetramer. Similarly, the CMT1B mutation Ser 49→Leu occurs at a residue whose side chain is normally packed against those of residues that are thought to mediate the intermembrane side-to-side adhesive interactions between apposed P_0 ex tetramers. All of these mutations are dominant, as would be predicted for changes that perturb interactions in a multimeric assembly.

At a few positions, differing mutations result in disease phenotypes of differing severity. Deletion of Ser 34, for example, is predicted to disrupt the in (hydrophobic) – out (hydrophilic) structure of a β -strand, and thereby to yield an unstable protein that might never be transported into the myelin organelle. This mutation, and other early nonsense mutations, result in a mild CMT1B neuropathy that is not dissimilar to the phenotype of aged P_0 heterozygote mouse knockouts¹⁰. But changing the same residue to Cys 34 generates an outwardly pointing thiol group that might be predicted to form nasty disulphide aggregates in the extracellular space. This Ser 34→Cys mutation results in the more severe DSS⁴.

Although detailed examination of the

severity and position of many CMT1B and DSS mutations is very satisfying in light of the P_0 ex crystal structure, this is not so for all known P_0 ex mutations⁴. It should be noted, however, that the genes encoding other less abundant myelin proteins, such as peripheral myelin protein 22 and connexin 32, are also subject to a variety of dominantly acting, dosage-sensitive mutations that result in peripheral neuropathies of varying severity⁹. For example, mutations associated with the PMP22 gene — especially interstitial chromosomal duplications and deletions that alter its dosage — are much more commonly observed in inherited human peripheral neuropathies than are mutations in the P_0 gene¹¹. It is therefore tempting to speculate that the actual macromolecular assembly in each myelin membrane consists not of complexes of a single protein, but rather of several proteins in addition to P_0 . The hole in the middle of each tetrameric P_0 ex doughnut might very well accommodate at least a portion of one of these other important myelin-specific proteins. □

Greg Lemke is in the Molecular Neurobiology Laboratory, Salk Institute for Biological Studies, La Jolla, California 92037, USA.

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Elevated art

WHAT is art? The question has defeated generations of critics. Daedalus now proposes a scientific criterion. A polished work of art, such as a poem, is an aesthetic optimum. Change any word, and the result is worse. By contrast, an everyday piece of journalism is not seriously damaged, and may even be improved, by changing a word here and there (which is why we have editors). Furthermore, says Daedalus, a truly great work of art is a sharp optimum. All its elements have been exactly and lovingly chosen and placed to combine in phase, as it were, to maximize its impact. Even a small alteration does serious damage.

This criterion deals harshly with much contemporary art. Free-verse poems, junk assemblies, dissected animals and human ejecta are very low, broad aesthetic optima even if they are optima at all. They could be altered in many ways without making them significantly worse.

On this view, the artistic process is a form of hill-climbing optimization. From a promising point in aesthetic space, the artist struggles through many small steps and occasional giant leaps, always climbing towards an envisaged optimum, with only luck or genius to guide him.

Daedalus has a new guide. The ultimate value of art is its emotional impact on the beholder. This can be measured in many ways — galvanic skin response, heartbeat, EEG spectrum, pupil dilation, and so on. Even that frisson of delight down the back of the spine could be captured by piezoelectric sensors. So Daedalus is wiring up a panel of selected critics and aesthetes to a sort of glorified lie-detector to monitor all these emotional responses. He will expose the panel to a range of acknowledged masterpieces, to establish the physiological 'signature' of great art. He will then offer them as a feedback guide to artists. Working under the gaze of the calibrated critics, and studying their changing instrumental reactions, the artist will shape his growing masterpiece to maximize its aesthetic impact on them. If he uses a computer — word or music processor, painting program or virtual-reality simulator — he could simply outline an initial sketch, and let an optimization package do the rest. It should soon find the ideal realization.

This simple process will probably never produce really great art. But it should splendidly meet the huge modern demand for second-rate art. Videos, pop music, commercials, posters, cartoon strips, pornography, packaging, jingles, displays and product styling could all be churned out in quantity with far less agony — except for that imposed on the hapless calibrated panel. David Jones

Penguin dispersal after fledging

SIR — Emperor penguins have several attributes that place them in high regard with biologists and others visiting Antarctica. The Ross Sea birds are of special interest because they occur in the last marine frontier that has not been exploited by humans, where biologists have the unique opportunity to do population studies before the pristine conditions are disrupted. Also, the geography of this sea provides a safe haven compared with areas on the outer perimeter of the continent. Two of our purposes in studying these birds were to determine: (1) if the initial foraging range of juveniles overlapped with pre-moult feeding adults, potentially resulting in competition; and (2) if the emperor penguin is to be an effective resource

detector of the Antarctic marine habitat, as proposed earlier¹, then we must know the boundaries of the habitat during all phases of its life cycle. One of the greatest challenges is to determine the needs of the recently fledged juveniles.

Pre-nuptial behaviour of emperor penguins begins on newly formed sea ice in April. From mid-May, when egg laying begins, until mid-July to early August, the male incubates the egg. At hatching the female returns to relieve the male, enabling him to break his 115-day winter fast²⁻⁵. During the next 5 months the parents share feeding of the rapidly growing chick, which weighs about 12 kg when it fledges around the time of the summer solstice. The entire breeding cycle takes

9 months. Shortly before fledging, the juveniles are left to fend for themselves. Once they leave the colony, they are seldom seen, and do not return again for several years^{5,6}.

In 1995 and 1996, using satellite transmitters, we tracked five juveniles for several weeks after departure from their natal colony. The results are surprising (see figure). The juveniles travelled beyond the Ross Sea, and as far north as 56.9° S. On 6 March 1996, the date of the last transmitter reception, one juvenile was 2,845 km from Cape Washington. Five of six birds studied left the Ross Sea and followed similar paths. They all travelled north to the Antarctic Convergence, the boundary of the Southern Ocean⁷, and then seemed to travel east, with the prevailing current and wind of the Westwind Drift. At these low latitudes the juveniles must be in ice-free waters and immersed at all times, a very different situation from that of the adults, which travel and hunt in ice-congested waters of high latitudes where they can rest frequently on ice floes.

Because their parents remain within the Ross Sea, the findings show that juveniles are not competing for the same food resource. We conclude that the critical

habitat of emperor penguins breeding in the Ross Sea is much larger than that sea alone. The extensive travel of the juveniles implies that Ross Sea populations of this species are, at least during one crucial part of their life, exposed to some of the same risks as more northern conspecifics. Of particular concern is the possible impact of commercial fishing around the Antarctic continent. We suggest that if adequate protection is to be given to this species, and perhaps to other Antarctic species as well, the dispersal patterns of the young must be determined. As written, the Antarctic Treaty and the Convention on Antarctic Marine Living Resources extend only to the 60th parallel in this region of Antarctica⁷. These new data suggest that the 60th parallel is probably too limiting to protect adequately even the most familiar symbol of Antarctic wildlife, the emperor penguin.

Gerald L. Kooyman

Tory G. Kooyman

Markus Horning

Carsten A. Kooyman

Scripps Institution of Oceanography,

University of California, San Diego,

La Jolla, California 92093-0204, USA

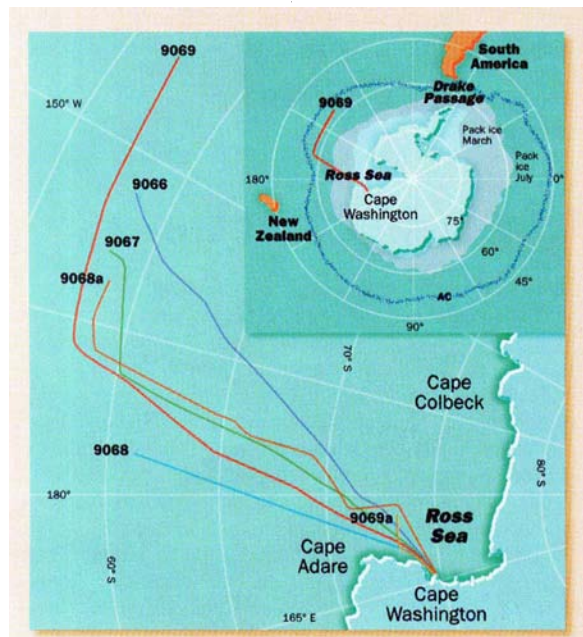
e-mail: gkooyman@ucsd.edu

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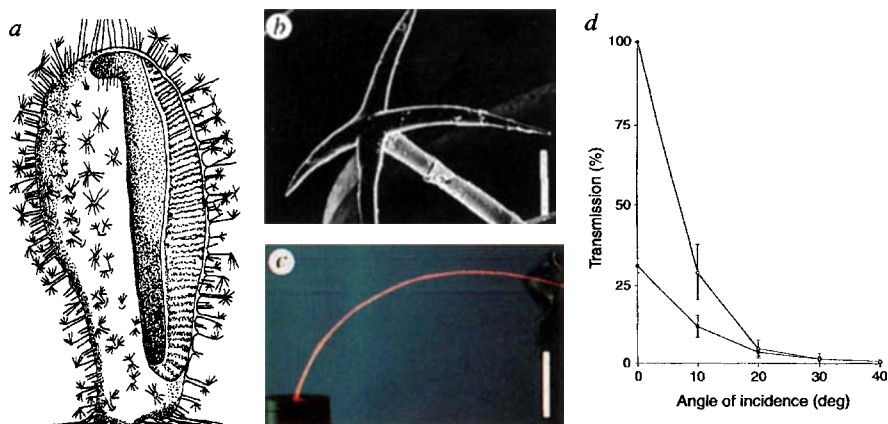
Optical fibres in an Antarctic sponge

SIR — The skeleton of demosponges and hexactinellids is generally composed of spicules consisting of amorphous hydrated silica (opal)¹ laid out around a proteic axial filament². Spicules constitute a mechanical support and act as deterrents to predators³. The presence of a filamentous green alga (*Ostreobium*) closely associated with the spicule bundles of a demosponge (*Tethya seychellensis*) suggested to us that light energy might reach the inside of the sponge body via siliceous spicules⁴. Such a natural optical-fibre system could influence sponge/symbiont interactions⁵⁻⁷ and wider aspects of sponge biology⁸.

We showed that siliceous spicules can act as optical fibres by using long spicules of an Antarctic hexactinellid (>10 cm long), whose large size allows easier handling of the very small demosponge



Routes of emperor penguin juveniles obtained from satellite transmitters (Telonics, ST-10; mass 120 g) attached by glue (Loctite 422) to the lower part of the back. From 15 to 19 December 1994 (9068a, 9069a) and 1995 (9066, 9067, 9068, 9069), the birds ranging in body mass from 10.9 to 14.2 kg (mean 12.8 kg $\pm$ 1.3 s.d.) (AND-FW 150 kg $\pm$ 20 g, platform scale) were captured and released near the ice edge of Cape Washington (74° 32' S; 165° 22' E). Within a few hours of release, the birds entered the water. Positions were monitored from 4 January 1995 (9069a) to 6 March 1996 (9069). During this time all birds had reached positions far enough north to be in the Westwind Drift⁸. We expected transmissions to continue during June, but the lack of signal suggests that the birds remained in water north of the pack ice. (Transmitter position on the bird prevents transmissions while the birds are swimming.) The pack-ice edge in this sector of the Southern Ocean based on satellite images was well south of 66° S by 7 December 1995. In the previous year, the pack ice edge on 15 June 1994 determined by direct observation by GLK from a US Air Force KC-10 flying at 11,000 m was at 61° S. General boundaries of the Antarctic Convergence and winter and summer pack-ice edges vary from year to year. Rendering of the inset is based on various figures^{7,8}. Antarctic Convergence (AC, dark blue line); pack-ice edges in July and March 1987 are shown.



a, *Rossella*, from a drawing by Schultze (ref. 10). b, Magnification of the cross-shaped apex of a *Rossella* spicule. Bar, 1 mm. c, A spicule bent to a 90° angle conducts red light from the source to an exposure meter Profisix (Gossen) modified to achieve light-spot measurement. Bar, 2 cm. d, Transmission of red light (635 nm) along spicules at different angles of incidence of the laser beam. Measurements were taken, at 30 mm from the apex, from spicules both with (upper plot) and without (lower plot) their cross-shaped apex. For each spicule, the transmission of light is expressed as a percentage of the value measured on the intact spicule aligned with the laser beam. Data represent the means of 10 measurements.

spicules. In the genus *Rossella*, in which there are species 1 m high (part a in the figure), these spicules protrude outwards from the sponge surface with a cross-shaped apex (b in the figure); the spicules are composed of a very compact opaline inner core and of a multi-stratified outer layer, rich in organic components².

We used pentactinal spicules from *Rossella racovitzae* Topsent 1901 (Porifera: Hexactinellida), sampled at 120 m depth in Terra Nova Bay (Ross Sea, Antarctica) during the PNRA XI Italian Antarctic Expedition (1994–95), to test for optical-fibre properties. We used a Sanyo compact laser beam unit, emitting red light (635 nm) (c in the figure) and carried out quantitative measurements on spicules technically bent to a 90° angle, with their cross-shaped apex aligned with the laser beam. The intensity of the conveyed light immediately after the bend, expressed as intensity measured at 25 mm from the cross-shaped apex, reached 65% after 10 mm (35 mm from the apex), decreasing to about 10% after 40 mm (65 mm from the apex).

To verify the role of the cross-shaped apex in conveying the light, we measured the values of light transmission at different angles of incidence to the laser beam

both on intact and on apex-deprived spicules (d in the figure). In both kinds of spicules, we detected light transmission up to a 40° angle of incidence to the laser beam, even though spicules deprived of

their cross-shaped apex transmit 70% less light than when the spicule is aligned with the beam. This result suggests that a larger light-capturing surface, as with the cross-shaped apex, enhances light transmission.

Scanning electron microscopy observations on *Rossella racovitzae* show that numerous intact diatoms adhere to its spicules, thereby suggesting the development of shade-adapted diatoms inside this deep-living sponge, as observed in Antarctic demosponges⁹. We believe that diatom survival in these peculiar environmental conditions is helped by this light transfer by siliceous spicules.

R. Cattaneo-Vietti, G. Bavestrello, C. Cerrano, M. Sarà

Istituto di Zoologia dell'Università di Genova,

Via Balbi 5, I-16126 Genova, Italy

U. Benatti, M. Giovine

Istituto Policattedra di Chimica Biologica dell'Università di Genova,

Viale Benedetto XV 1,

I-16132 Genova, Italy

E. Gaino

Istituto di Zoologia dell'Università di Perugia,

Via Elce di Sotto, I-06123 Perugia, Italy

Distant gene flow in tropical trees

SIR — Canopy trees, the main structural elements of tropical rain forests, typically occur at the density of one adult or less per hectare^{1–3}. Because these trees are strongly outcrossed^{4,6}, their populations are expected to cover large areas of hundreds of square kilometres. These large populations are now being fragmented by deforestation and habitat alteration, but the genetic consequences of fragmentation remain largely unexplored because of the lack of information about effective population sizes and gene flow in canopy trees^{7,8}. We have recently succeeded in isolating a relatively new class of genetic markers, simple sequence repeats (SSRs), in tropical trees and have demonstrated their use in addressing central issues in conservation biology⁹. Here, we demonstrate the use of these markers in estimating gene flow and mating parameters in *Pithecellobium elegans*, a large canopy tree from central American rain forests. Our measurement of the distance of pollen flow is among the largest, and exclusion probabilities for assigning paternity the highest reported for any natural population of plant species. Furthermore, our methods allow us to assign paternity to almost any seed collected from the population.

We studied gene flow and mating patterns among 28 adult trees in a forest 'fragment' 7 km west of La Selva Biological Field Station in the Atlantic lowlands of Costa Rica. The 'fragment' (Chilamate) was converted to pasture approximately

15 years ago, but the original trees of *P. elegans* were retained. One edge of the forest 'fragment' (Fig. 1) is separated by less than 30 m from natural, but degraded, forest.

We characterized nine SSR loci in *P. elegans*. Allelic diversity in this population ranges from 5 to 14 alleles per locus for the 28 trees in this site. For this study we used the following five loci: *Pel2* (5 alleles); *Pel3* (6 alleles); *Pel5* (5 alleles); *Pel6* (12 alleles); and *Pel7* (14 alleles). The high allelic diversity and the relatively large number of loci allowed us to identify unambiguously the pollen parents of individual seeds. All adults were uniquely genotyped from DNA isolated from leaves. To identify actual pollen parents and to estimate pollen flow, we analysed 167 seeds from progeny arrays of six trees. Each seed does not represent an independent mating event, because our analysis of paternity revealed that seeds within a pod generally have a single pollen parent (see below). Out of 167 seeds, 110 seeds were from 15 separate pods and the remainder represent seeds from 57 independent mating events.

Two critical attributes of this study allowed for an extremely precise description of the gene flow patterns in *P. elegans*. First, all trees were identified, mapped and genotyped. Second, and more important, the SSR loci provide the highest exclusion probabilities of any set of markers used in any study of natural plant

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populations. The 42 alleles used here give better than a 99% exclusion probability. Thus, we are not restricted to the use of rare alleles and selected individuals, but can examine random pollination events at any location in the population to examine the pollen flow dynamics and population structure. These data are used to characterize mating events at the level of the fruit, the individual tree and the population.

In many outcrossing species there is a substantial degree of pollen competition, and progeny arrays from single fruits are generally half-sibs. Several species in the Leguminosae are known to shed pollen as polyads consisting of up to 32 pollen grains (16 in *P. elegans*) derived from a single meiotic event. A single deposition by a pollinator can possibly ensure full seed production. Of 24 pods examined (15 from Chilamate and 9 others from La Selva), only two showed multiple paternity (two different fathers). Of these two, one had 18 seeds, thus requiring at least two polyads. There was no significant segregation distortion for either the male or female parent of these mating events.

We identified a paternal genotype or haplotype for all 72 mating events. The identification of a single father or the exclusion of all fathers was possible for 70 (97.2%) mating events. Of these 70, 41 (58.6%) of the pollen donors were from the genotyped population and 20 (28.6%) were clearly not from this marked population. Another 9 of the 70 mating events

(12.8%) were either the result of selfing or from a genetically identical pollen donor outside the marked population. These 9 events are progeny from a single individual. Finally, 2 of the 72 matings (2.8%) remain unresolved because the paternal haplotypes could have been derived from two different trees in the mapped population.

These mating events showed that gene flow from known fathers averaged 142 m in our study site (Fig. 2a). This large mean distance of pollen transfer contrasts starkly with the average distance of the nearest neighbours in the population (27 m). A typical leptokurtic distribution of pollination events was not evident in this population. Although one might expect matings to be among neighbouring individuals, pollen contributors to most of the seeds analysed were greater than 10 trees away (Fig. 2b). An obvious explanation for this result is variation in phenology. Flowering is episodic; although some individuals may dominate the pollen pool with massive flower production in some years, they may be minimal contributors to the pool in other years. Thus, the effective population may be considerably smaller than the number of individuals in the site but may cover large physical distances.

The 142 m average gene-flow distance is probably an underestimate of the true gene flow in the population, as our data indicate that many of the mating events are the result of gene flow from outside the site. The longest gene-flow distance covered is approximately 350 m, which may be the longest pollen-flow event precisely characterized in any species.

This species of tree is pollinated by hawkmoths, known to forage over long distances (W. A. Haber, personal communication), as is the case with other common pollinators of tropical trees such as bees^{10,11} and bats¹². Not surprisingly, at least 29% of the matings are from pollen sources outside the marked population.

Mating patterns in plants follow a hierarchical organization. We have shown for the first time how the allelic diversity at SSR loci can be used to characterize mating events at the level of fruit, individual and subpopulation. The most surprising result is that, in contrast to other species^{13–15}, most mating events in this species are not with the closest neighbours. Thus forest fragments, as well as isolated trees in pastures, may be stepping stones for gene flow among patches

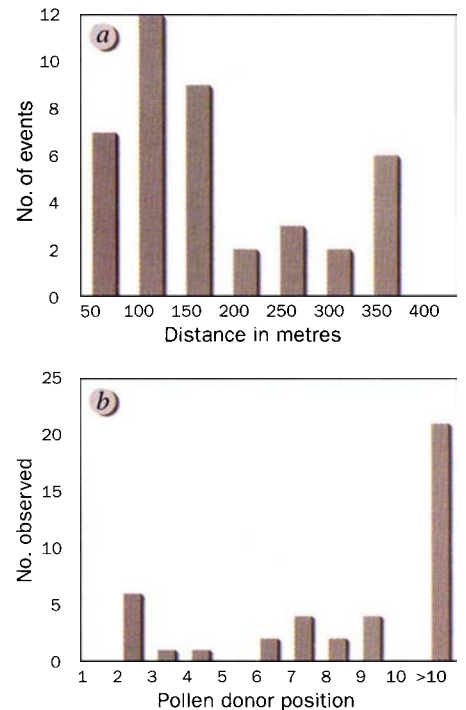


FIG. 2 Relative pollen flow in the Chilamate population of *P. elegans*. The nine probable selfing events are not included. a, Physical distances of pollen sources from the seed parent; b, relative neighbourhood position of the pollen parent. For all defined mating events, the relative position of the pollen donor to the seed plant was determined. The position could range from 1, indicating self-fertilization, to greater than 10, indicating that at least 10 other adults were closer to the seed plant than the pollen donor.

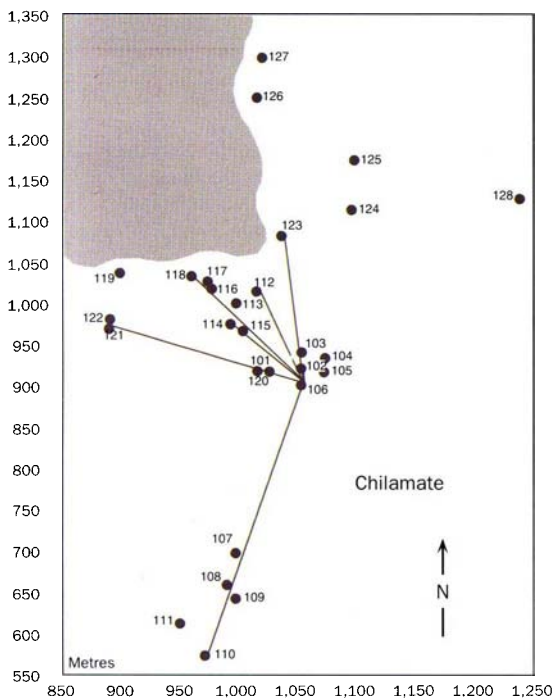


FIG. 1 Physical map of the population of adult *P. elegans* trees and pollen flow events detected to tree 106. Distances estimated with the aid of a rangefinder. Unknown reproductive individuals may exist in partially degraded natural forest (shaded area).

which contribute to the genetic diversity of contiguous undisturbed forests.

**M. R. Chase, C. Moller
R. Kesseli & K. S. Bawa***

Department of Biology
University of Massachusetts, Boston,
100 Morrissey Blvd,
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*To whom correspondence should be addressed.

HIV blocked by chemokine antagonist

SIR — The recent discoveries that chemokine receptors are cofactors for HIV entry into CD4-bearing lymphocytes¹, and that CC chemokines have anti-HIV effects², have markedly changed our views on HIV infection and AIDS. The CC chemokines RANTES, MIP-1 α and MIP-1 β inhibit infection of lymphocytes with macrophage-tropic HIV isolates by interaction with the CCR5 receptor³, and the CXC chemokine SDF-1 was recently identified as the ligand for CXCR4 (formerly called fusin, LESTR and HUMSTR) and shown to block infection with T-cell tropic HIV isolates^{4,5}. Together, these observations suggest that chemokines or chemokine derivatives may be useful agents for the treatment of infection with HIV.

Therapy based on administration or overexpression of chemokines, however, may be hampered by the chemotactic and activating effects of these powerful mediators on leukocytes. For this reason, we have studied the anti-HIV effects of a derivative of RANTES which lacks chemotactic and leukocyte-activating properties and acts as a receptor antagonist. We synthesized the antagonist as an analogue of RANTES with the first eight amino-terminal amino acids deleted and termed it RANTES(9-68). This analogue is known to bind multiple CC-chemokine receptors and to inhibit chemotaxis, Ca²⁺

mobilization and enzyme release⁶. Figure 1 shows that RANTES(9-68) lacks agonistic activity on phytohaemagglutinin (PHA)-treated and interleukin-2 (IL-2)-expanded peripheral blood mononuclear cells and inhibits Ca²⁺ mobilization induced by RANTES. In addition RANTES(9-68) is a potent inhibitor of infection with macrophage-tropic HIV.

Like RANTES, the antagonist does not affect the replication of the lymphocyte-tropic HIV strain LAI (Fig. 2). The anti-HIV activity of the antagonist is somewhat lower than that of RANTES itself, which is in keeping with its lower affinity for CC chemokine receptors as previously described⁶. Inhibition of viral replication is concentration dependent, exceeding 90% at 30–300 nM RANTES(9-68) and 3–30 nM RANTES. As indicated by the range of effective concentrations, the anti-HIV activity of the antagonist as well as that of RANTES shows some variability, depending on the donor of the cells.

In several experiments with cells from different donors, we never observed enhanced replication of macrophage-tropic HIV isolates in human macrophage cultures treated with RANTES or the RANTES antagonist (data not shown). Note, however, that the use of antagonists instead of wild-type chemokines will avoid side effects that may result from agonistic

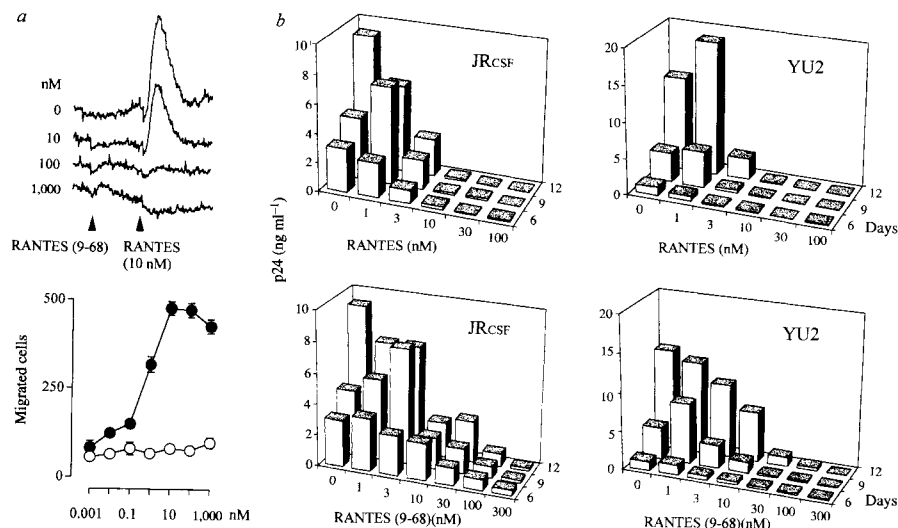


FIG. 1 a, PHA-activated peripheral blood mononuclear cells were cultured for 10 days in medium containing interleukin-2 (IL-2), and then tested for [Ca²⁺]_i changes and chemotaxis. We recorded [Ca²⁺]_i-dependent fluorescence changes in Fura2/AM-loaded cells after addition of increasing concentrations of RANTES(9-68) followed, after 60 s, by 10 nM wild-type RANTES. Tracings show that RANTES(9-68) lacks agonist activity and inhibits the response of the cells to RANTES. Chemotaxis in response to RANTES (filled circles) or RANTES(9-68) (open circles) was performed in 48-well chambers with 105 cells per well using collagen-coated polyvinylpyrrolidone-free polycarbonate filters with 3- μ m pores. Migrated cells were counted in five randomly selected fields at 1,000-fold magnification after migration for 1 h. b, PHA-activated peripheral blood mononuclear cells from two healthy donors were infected with 50 half-maximal tissue-culture infectious dose units (TCID₅₀) of either the JRCSF (donor 1) or YU2 (donor 2) NSI primary HIV-1 isolates. Immediately after infection, RANTES or the antagonist RANTES(9-68) were added to cultures at the indicated concentrations. Cultures were supplied with IL-2 and RANTES or RANTES(9-68) every third day. Production of soluble HIV-1 p24 was monitored in the culture supernatant as an indicator of HIV replication, and the data for day 6, 9 and 12 are shown.

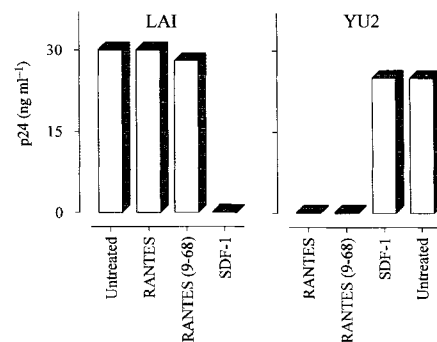


FIG. 2 PHA-activated peripheral blood mononuclear cells from healthy donors were infected with 50 TCID₅₀ of either LAI or YU2 HIV-1 isolates. Immediately after infection, 100 nM RANTES, 1,000 nM RANTES(9-68) or 100 nM SDF-1 were added. Cultures were re-fed every 3 days with IL-2 and chemokines, and soluble HIV-1 p24 was determined after 12 days.

activity, for example as recently reported in Scientific Correspondence, RANTES, MIP-1 α and MIP-1 β enhance HIV replication in macrophages and virus-induced inflammation⁷.

Our findings show that structural modification of a chemokine can yield a variant lacking chemotactic and leukocyte-activating properties, but still retaining high affinity for chemokine receptors and the ability to block HIV infection. This indicates that receptor signalling and cell activation is probably not required for the anti-HIV effect of chemokines, and that blockade of the receptor may be sufficient. Owing to the lack of agonist effects, CC and CXC chemokine antagonists are unlikely to elicit inflammation, affect leukocyte trafficking or enhance HIV replication, actions that would restrict their potential use as antiviral substances in HIV-infected patients. The development of such therapeutic agents is encouraged by the present results.

Fernando Arenzana-Seisdedos

Jean-Louis Virelizier

Dominique Rousset

Unité d'Immunologie Virale,

Institut Pasteur,

75724 Paris Cedex 15, France

Ian Clark-Lewis

Biomedical Research Centre,

University of British Columbia, Vancouver,

BC V6T 1Z3, Canada

Plus Loetscher, Bernhard Moser

Marco Baggiolini

Theodor Kocher Institute,

University of Bern, PO Box 3000,

Bern 9, Switzerland

e-mail: baggiolini@tki.unibe.ch

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Discovery of hardest known oxide

SIR — Microhardness measurements on synthesized samples of stishovite, a high-pressure phase of silica, show that it is the hardest oxide yet discovered. Among polycrystalline materials, its hardness (33 gigapascals, GPa) rivals those of the hardest materials. Despite many searches¹⁻⁴, no material with a measured hardness comparable to diamond or cubic boron nitride has been identified until now.

Hardness (H) of ionic and covalent materials increases with bulk modulus¹⁻⁵. Diamond has the highest known bulk modulus, $B = 444$ GPa, and is also the hardest material known, with single-crystal $H = 90$ GPa. It is followed by cubic boron nitride (cBN), with corresponding values of $B = 369$ GPa and single-crystal $H = 48$ GPa (refs 2, 4).

Theoretical calculations have been used in this search for hard materials, principally by looking for high-bulk-modulus compounds. A semi-empirical theory¹ was applied to group IV elements and III-V and II-VI compounds: $B = N/4 (1,971-220I)d^{-3.5}$ (where N is the average coordination number, I is an empirical ionicity parameter and d the bond length). However, no superhard material has been synthesized as a result of these calculations and the stability of the predicted compounds has not been verified.

Under ambient conditions, the bulk modulus of ionic compounds is given by a general relationship: $B \propto Z_a Z_c / V$, where Z_a and Z_c are the formal anion and cation charges respectively, and V is the specific volume per ion pair⁶. High bulk moduli require high charges and small volumes; thus tetravalent cation dioxides could be hard. Under pressure, packing efficiency increases at phase transitions at which the cation coordination number (N_c) rises. As structures become more compact, compression becomes increasingly difficult and the bulk modulus rises. High-pressure phases may thus be hard materials. The transformation from graphite to diamond is the archetypical example.

Although silicon is the smallest tetravalent cation, the common forms of silicon dioxide are not hard because of their open structures in the cristobalite or quartz phases ($N_c = 4$). However, the high pressure phase⁷ of silica, stishovite ($N_c = 6$), could potentially be a hard material. It is much denser than quartz,

and is metastable under normal conditions⁷; its bulk modulus, 298 GPa (ref. 8), is significantly greater than that of alumina, 252 GPa, which is itself a hard oxide. Here, we report microhardness measurements on synthesized stishovite.

We performed synthesis experiments using a 1,200-ton uniaxial MA-8 multi-anvil apparatus (see ref. 9 for method), taking Raman and X-ray diffraction (see ref. 10 for image plate system) measurements. Microprobe analysis showed only silicon in the investigated region. We did the hardness tests, using a Knoop microhardness tester (Shimadzu type M) with

Bulk moduli and Knoop hardness for polycrystalline hard materials

Material	B (GPa)	H (GPa)
B ₄ C	200	30
B ₆ O	200	30
SiC	248	29
Al ₂ O ₃	252	21; 19*
SiO ₂	298	33*
Sintered cBN	369	32
Sintered diamond	444	50

Values from ref. 4, except for B₆O (ref. 12) and asterisks (this work).

loads of 490, 980 and 1,960 mN, on quenched samples after polishing. The hardness values obtained are independent of load and those of a test sample of alumina are in excellent agreement with values cited in the literature (see table).

We treated a sample of α -quartz at 14 GPa and 1,000 °C; X-ray diffraction indicated that the sample was >99% stishovite. Micro-Raman spectroscopy indicated the presence of coesite and possibly quartz (line around 490 cm⁻¹) at various places on the sample surface; the lines of stishovite were observed in all cases. The hardness measurements ranged between 19 and 33 GPa. This scatter was attributed to the presence of small amounts of quartz or coesite, both of which have very low hardness, on the surface of the sample, as revealed by the Raman spectroscopy.

To test this hypothesis, we synthesized another sample at higher pressure: 20 GPa and 1,100 °C, using amorphous silica as the starting material. The transformation to polycrystalline stishovite was complete and we could detect no foreign phase by Raman spectroscopy or X-ray diffraction. The seven indentation measurements indicated a very high hardness ranging from 30.9 to 34.7 GPa, with an average value of 33 GPa, irrespective of the orientation of the indenter and the load. We noticed no change in the Raman spectrum before and after indentation.

The second experiment definitely shows that stishovite is very hard. The

hardness of stishovite has been reported to be 17–20.8 GPa along different directions⁷, but the sample used in this case had been synthesized at about 9.5–10 GPa and 1,200–1,400 °C. The transformation was not complete at this pressure, as later shown¹¹. The small amount of lower-pressure phases present drastically modified the indentation results.

Polycrystalline stishovite is now the hardest oxide known; it is harder than alumina and boron oxides¹². Other superhard polycrystalline materials include diamond and cubic boron nitride compacts; their hardness is much lower than that of single crystals, with hardness values of 50 and 32 GPa, respectively⁴. Thus, stishovite is among the hardest polycrystalline materials known.

Stishovite is thus potentially an important technological material. It may be the first member of a new family of superhard materials: the metastable high-pressure phases of high-valence cation oxides with high bulk moduli induced by an increase of the coordination number.

J. M. Léger

J. Haines

Laboratoire de Physico-Chimie des Matériaux,

CNRS, 1 Place Aristide Briand, 92190 Meudon, France

M. Schmidt*

Bayerisches Geoinstitut, D-95440 Bayreuth, Germany

J. P. Petit

Laboratoire d'Ingénierie des Matériaux et des Hautes Pressions, CNRS, Avenue J. B. Clément, 93430 Villetaneuse, France

A. S. Pereira

Escola de Engenharia e Instituto de Física, UFRGS, 91501-970 Porto Alegre, RS, Brazil

J. A. H. da Jornada

Instituto de Física, UFRGS, 91501-970 Porto Alegre, RS, Brazil

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*Present address: CNRS, URA10, Université Blaise Pascal, 63038 Clermont-Ferrand, France.

Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words.

Chlorophyll *d* as a major pigment

SIR — Chlorophyll *d* was reported first as a minor, green and magnesium-containing pigment in various species of red macroalgae in 1943 (ref. 1). It was thought by some, however, that chlorophyll *d* could be an artefact produced by the pigment extraction process, as it is not found in all algae, and one of the oxidation derivatives of chlorophyll *a* has an absorption spectrum identical with that of chlorophyll *d*^{2,3}. It remains unclear as to whether chlorophyll *d* does occur *in vivo*. We have now isolated a previously undescribed oxygenic photosynthetic prokaryote containing chlorophyll *d* as a major green pigment: it has only a small amount of chlorophyll *a*.

We isolated the new organism from a suspension of algae squeezed out of *Lissoclinum patella*, a colonial ascidian, collected in 1993 from the marine coast of the Palau islands in the western Pacific Ocean. Cells are unicellular and spheroidal or ellipsoidal, 1.5–2.0 µm in diameter and 2.0–3.0 µm in length. They are photoautotrophs, and have evolved in the presence of oxygen. We used electron

microscopy to demonstrate that the cell is a prokaryote (Fig. 1a, b). About 10 to 15 layers of thylakoid-like membranes are stacked peripherally (Fig. 1b), and we observed no protein entities similar to phycobilisomes on the membranes (Fig. 1a). These ultrastructural features are very similar to those of the Prochlorophyta (Oxychlorobacteria), oxygenic photosynthetic prokaryotes, containing chlorophylls *a* and *b* without phycobiliproteins, rather than those of the Cyanophyta (Cyanobacteria), containing chlorophyll *a* with phycobiliproteins.

The cell suspensions are green and have an absorption maximum in the red region at 714–718 nm (Fig. 1c), whereas all known oxygenic photosynthetic organisms have a characteristic absorption maximum around 675 nm due to a bathochromic shift of chlorophyll *a*⁴. Pigment analysis by HPLC reveals that a green pigment with three absorption maxima at 400, 455 and 697 nm in methanol is a predominant pigment of the cell in addition to only a small amount of chlorophyll *a*. The absorption spectra of the predominant pigment and of its acidification product (peaks at 392, 421 and 692 nm in diethyl ether) are the same as those reported for chlorophyll *d* and phaeophytin *d*¹, respectively. We confirmed that the predominant pigment is chlorophyll *d* by NMR and fast atom bombardment mass spectrometry (Fig. 2).

The chlorophyll *d* makes up about 80% of the total lipid-soluble pigment of the cell and more than 2% of the cell dry weight. The amount of chlorophyll *a*, on the other hand, is less than 0.08% of the cell dry weight, whereas it ranges from 0.3% to 3% in other oxygenic photosynthetic organisms⁵. The ratio of chlorophyll *a* to chlorophyll *d* in the

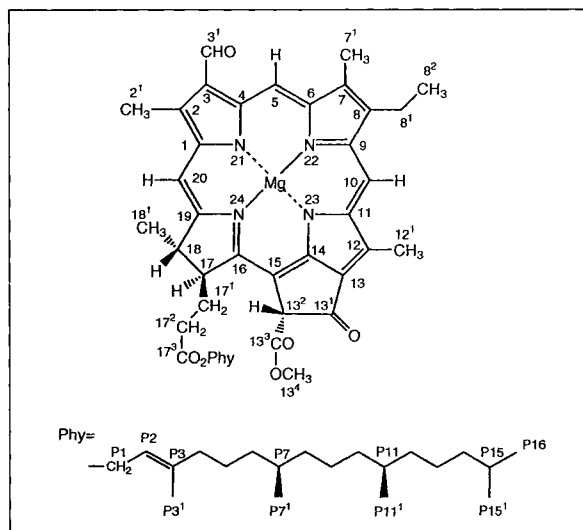


FIG. 2 Structure of chlorophyll *d*.

cells varies between 0.027 and 0.092, depending on the culture conditions.

Our results confirm the natural occurrence of chlorophyll *d* in a newly isolated, oxygenic, photosynthetic prokaryote. Moreover, the higher content of chlorophyll *d* and the low ratio of chlorophyll *a* to chlorophyll *d* in this organism suggest the existence of a unique and as yet unknown light-harvesting system which uses chlorophyll *d*. We propose for the new organism the name *Acaryochloris marina* Miyashita et Chihara gen. et sp. nov., to be formally described elsewhere (manuscript in preparation).

Hideaki Miyashita

Hisato Ikemoto

Norihide Kurano

Marine Biotechnology Institute,
Kamaishi Laboratories,
Kamaishi,
Iwate 026, Japan

Kyoko Adachi

Marine Biotechnology Institute,
Shimizu Laboratories,
Shimizu,
Shizuoka 424-19, Japan

Mitsuo Chihara

Japanese Red Cross College
of Nursing,
Shibuya-ku,
Tokyo 150, Japan

Marine Biotechnology Institute,
Kamaishi Laboratories
Kamaishi,
Iwate 026, Japan

Shigetoh Miyachi

Marine Biotechnology Institute,
Hongo, Bunkyo-ku,
Tokyo 113, Japan
e-mail: miya@mb.sendai.infoweb.or.jp

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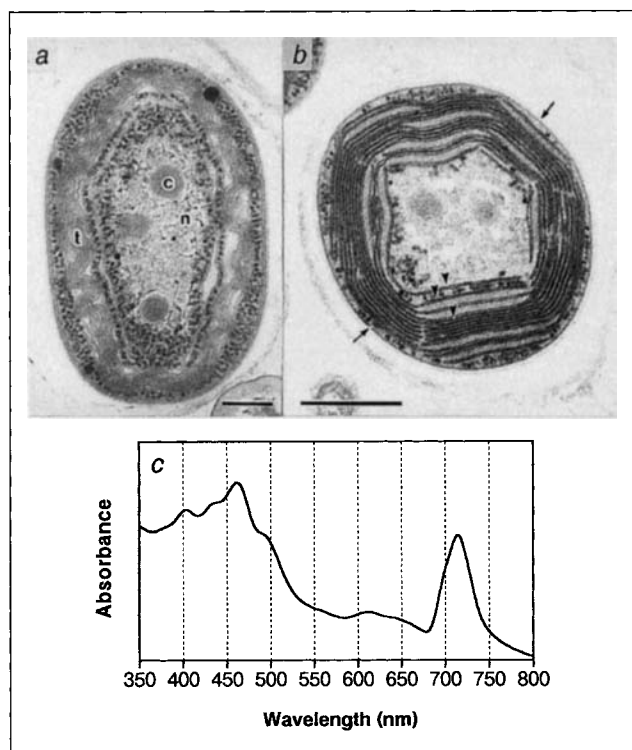


FIG. 1 a, Electron micrograph of a longitudinal section of a cell, showing the cytoplasmic space. The preparation was prefixed with 1% glutaraldehyde and postfixed with 2% osmic acid. c, carboxysome-like structure; n, nucleoid; t, thylakoid-like membranes. Scale bar, 0.3 µm. b, Electron micrograph of transverse section showing thylakoid-like membranes and plasmalemma. Cell prefixed with 1% glutaraldehyde and postfixed with 1% potassium permanganate. Arrowheads, thylakoid-like membranes; arrows, plasmalemma. Scale bar, 0.5 µm. c, Absorption spectrum of cells suspended in fresh culture medium.

Between a rock and a hard place

Anthony F. Aveni

Stonehenge: Neolithic Man and the Cosmos. By John North. *HarperCollins: 1996.* Pp. 609. £25.

OUR century has witnessed three distinct phases in the invasion of quantitative science into attempts at discerning ancient motives for erecting Stonehenge. It opened with the British astronomer Sir Norman Lockyer trying to pin a precise date to the construction of the monument through astronomical orientation. Discarded for more than half a century because it did not fit archaeological chronology, the astronomical hypothesis was resurrected by another astronomer, Gerald Hawkins, who proposed that the monument was a gigantic calculator for predicting lunar and solar eclipses. The engineer Alexander Thom went further, postulating that many of Britain's megalithic monuments were intended as precise observatories for charting the movements of the Moon with respect to prominent bumps and notches on the horizon, and proposing that their builders, who used a megalithic yard 0.829 metres in length, had a sophisticated knowledge of Pythagorean geometry well in advance of the Greeks.

Although the notion of Neolithic wise men with a religious passion for astronomy caught the public eye, surveys and analyses in the 1980s and 1990s by a new generation of interdisciplinary scholars signalled the start of a third interpretive phase that revealed that many of these earlier claims had been grossly overstated. Those who built Stonehenge were intent on following the two great luminaries between their respective horizon standstills and they surely had a rudimentary knowledge of how to lay out right-angled triangles, but neither of these enterprises did they undertake with the extravagant precision indicated by the engineers and astronomers of the 1960s.

John North's monumental book will come as a controversial throwback to the old astronomical framework. He claims that the religious make-up of Neolithic architects was imbued with a passion for astronomy so overriding that sky events were configured into the placement, shape, alignment — indeed into every bend, turn and asymmetry — of practically every artefact in the landscape. This devotion to astronomy, North contends, began with an interest in stellar orientation evident in the earliest remains and evolved into a fixation with the movement of the Sun and Moon between their horizon standstills, which he claims to discover in the alignments of later monuments.

North's treatment is chronological, beginning with an analysis of barrows or chamber tombs. He astutely mines the archaeological record and extant plans, concluding that as a class these show a preferential orientation in absolute space. But given their location miles apart, such a direction can have been established only with reference to celestial bodies — not to the Sun and Moon as had already been

ancient structure might be astronomically oriented — that is, investigating all the directions that could be indicated by the alignment of its parts and then applying probability considerations, North eliminates what he considers to be unlikely directions, such as lines between standing stones too close to one another to have been useful in a sighting scheme.

Having established his methodology, North then moves on to the cursuses (roughly parallel bank and ditch structures of kilometre lengths) and other enclosures. He contends that many of these incorporated a slope of 1:5 using Thom's megalithic yard as a standard unit of linear measure. According to North, the banks are crooked not because of sloppy construction but because they evolved over

IMAGE
UNAVAILABLE
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REASONS

Is it a wonder of geometry? Is it a window to the stars? Or is it just a load of old stones? Stonehenge in Wiltshire, UK, the centre of a great discussion.

proposed for Neolithic burials throughout Mediterranean Europe, but rather to the stars. He hypothesizes that the custom developed of aligning the barrows on pairs of bright stars simultaneously (for example, the Pleiades setting and Spica rising). These alignments, taken in different directions and over different elevations, account for barrow asymmetries such as tapering and vertical contour. North takes advantage of the changing rise-set positions of stars owing to the precession of the equinoxes to establish ranges of dates when his stellar pairs yield alignments consistent with the archaeological record.

Rather than following conventional methods for determining whether an

time as part of an astronomically determined plan. The cursuses were built up out of stellar-oriented straight sections linked together. Consideration of the third dimension is among the original aspects of North's theory of barrow and cursus construction. In this way, an observer stands in the ditch and looks over the edge of the bank that flanks it, the bank taking the place of an artificial horizon.

Many of the colossal figures cut into southern England's chalky turf, such as the famous Uffington white horse, the long man and the Cerne giant, acquire astronomical identity in a chapter of their own. Their creators were apparently seeking out the same effects of landscape elevation

and contour to accommodate the perceived celestial template. Viewed through North's eyes, each figure becomes a constellation in the oldest of all prehistoric star maps: the horse is Taurus (Aldebaran marks the penis rather than the eye), the long man is Hercules and the obscene giant is our Orion, whose celestial trek, viewed from the proper place, dramatically carries him over the downs on which his countenance is carved — "the Orion Effect".

A chapter on avenues argues that the *via sacra*, whether at Avebury or Stonehenge, is not excluded from the ever evolving astral religion. Nor are the earlier henge rings, which were made up of specific numbers of posts, not to accommodate a calendrical count, but because of the acceptable angles they threw up for alignment making. So the 56 Aubrey holes at Stonehenge (North insists they once held wooden posts despite the almost unanimous agreement among archaeologists that they never did) make accessible to the eye the periodic limits of swing of Sun and Moon better than a 51- or 61-post circle, provided one subjectively sights from backsight posts on one side of the circle to selected foresight posts on the other side, conveniently eliminating most of the other posts. What then, one wonders, was their function? Using only a ground plan, North arbitrarily converts post holes of different diameter to post lengths of proportionate height, assuming a fixed ratio of exposed to buried depth (in units of the megalithic yard), this for the purpose of viewing various astronomical phenomena in the interstices between posts and lintels. "What one must do next is juggle the arrangement of the lintels to create a convincing artificial horizon that fits with what is known of post hole sizes, especially depths, and the derived altitude." Convincing for whom, one wonders?

North's treatment of Stonehenge is a laborious, meticulous exercise in the art of placing megaliths to block out unwanted sight lines in order to produce the desired astronomical windows. The station stones reflect a continuation of the tradition of alignments relating to the earlier Aubrey circle; the slaughter stone emerges as a viewing device linked to the altar stone; and the celebrated heelstone was precisely positioned to produce the desired sky view.

There are several reasons why, when it comes to postulating theories of orientation, archaeoastronomers stay away from the stars. First of all there is the problem of selection—there are 6,000 points of light visible to the naked eye. North chooses the 20 or so brightest of them, as well as the Pleiades, because of their conspicuousness. But anthropologists tell us that the stars to which indigenous people pay the most attention are not necessarily

the most luminous, but those that happen to be in the right place at the right time — for example, among the Borana of East Africa the stars of our inconspicuous constellation of Triangulum are the most important ones in setting the lunar year. A second problem is that, unlike the Sun and Moon, the stellar changes in rise-set position are both highly dependent on the epoch and variable from star to star. Third is the so-called problem of extinction. Even under optimum conditions, only the two or three very brightest stars are visible all the way down to zero-degrees elevation, and the fainter the star, the higher above the horizon it will be seen to disappear. Despite his assertion that precise values of the extinction angles he uses are unimportant in most cases (because the natural or artificial horizon elevation exceeds the extinction angle), in those cases where they do matter, North does not refer to published observational and theoretical studies of extinction that reveal the highly indeterminate nature of this parameter. Operating in a frequently cloud-bound region and at high latitude, where the azimuthal shift for differential altitudes of appearance and/or disappearance is especially great, throws large error factors into the determination of alignments that are as strongly time-dependent as those used by North. For example, a change in the visual extinction coefficient (the number of stellar magnitudes of extinction suffered by starlight in the direction of the zenith) of a mere 0.1 magnitude from optimum will produce an azimuthal shift averaging about one degree for the brightest stars. This translates to a horizontal shift in the relative placement of a pair of opposing posts comprising a ring the size of Stonehenge's Aubrey circle of about 1.5 metres.

In other words, to assure an astronomical rendering, North manipulates the components of a monument to fit the stars; but given the astronomical subjectivity and uncertainty, together with the impossibility of reading from ordnance maps the precise gradients and heights of ditches dug in turf 5,000 years ago, making things fit is a bit like using a rubber template and blunt architect's pencil to sketch out a design plan.

The Stonehenge that North desires is a virtual cyberhenge—the ultimate in celestial determinism, the product of a highly reductive scientifically oriented intellect exploring sky knowledge as an end in itself. Those who erected it were master-planners with a profound knowledge of geometry and mensuration who not only used sketch plans but also carefully chose both the latitude and the landscape upon which they built their temples, tombs and access ways to conform to divine celestial dictates. What emerges from North's analysis is architecture made to look

through not *at*. Its pristine design, governed by a "geometry of obscurity", bears an uncanny resemblance to the Renaissance ideal of Inigo Jones.

Any reader who perseveres through North's tortuous argumentation and complex computer-generated diagrams of ideal henges should arrive at the last 30 pages of the book wondering about the 'why' of it all. Why would a prehistoric community indulge in rigorous astrogeometrical machinations rivalling Kepler in both persistence and obsession? And why continue and revise the process for millennia? To demonstrate merely that all these artefacts 'work' to the author's specifications as astronomical sighting devices, whether to store secret knowledge of the sky gods masquerading as dead ancestors or to serve as genuine observatories, is not sufficient. No theory of ancient society is complete unless it can be demonstrated to be consistent with whatever else the material record reveals about that society. But unfortunately there is precious little data to guide us when it comes to preliterate cultures, and the concluding chapter on "Ritual and Belief" does little to answer why. Instead, we acquire only generic knowledge: for example, sky religion became prominent at many different stages of religious evolution (most religionists would indeed quarrel with *any* evolutionary theory being applied to religion); gods of the sky often functioned as primary deities, "stellified ancestors", "surrogates for the soul of the dead"; and many cultures such as the Hopi and Nuer pay attention to important celestial directions.

What goes around comes around, whether the circuit be fashion in clothing, music or prehistory. North, whom I greatly admire as a historian of science, seems to have overstepped the confinement of his data boundary and fashioned an overdetermined panoply of astronomically oriented prehistoric monuments. This Stonehenge is too perfect and the people he has fashioned to erect it seem to emulate that old scientific priesthood conjured up at the turn of the century, computer graphics added. But today this sort of highly reductive scientific determinism rides a wave of popular enthusiasm. In the decade of the 1990s alone, the idea that the sky served as a template that guided the evolution of sacred architecture has been applied to the Egyptian and Mexican pyramids, the Inca capital of Cuzco, the giant geoglyphs on the Nazca desert and the Mithraic houses of worship of ancient Rome. Where will the next millennium take us? □

Anthony F. Aveni is in the Departments of Physics and Astronomy, and Sociology and Anthropology, Colgate University, 13 Oak Drive, Hamilton, New York 13346, USA.

Technology drive

Maurice V. Wilkes

Computer: A History of the Information Machine. By Martin Campbell-Kelly and William Aspray. *BasicBooks*: 1996. Pp. 342. \$28.

THE authors offer this work as a popular history of the computer. It starts with three chapters about what went on in offices before computers existed, with the authors pointing out quite properly that information processing did not start with computers. Then come three chapters on the work of the early pioneers of electronic computers leading to the development of the computer industry. The next group of three chapters contains a selective history of the main innovations that came between the end of the Second World War and the advent of the first microprocessors. The final three chapters trace the progress of microcomputers from around 1975, when they were mainly of interest to amateurs, to the arrival of the professional personal computer. By that time, software rather than hardware had become the important

purpose computer. I entirely fail to follow the authors when they state that the Analytical Engine would have served no purpose that the Difference Engine could not have satisfied and that no-one would have wanted it. Nor do I follow their speculations on the exact reasons why the British government of 1842 declined to revive the dormant Difference Engine project with fresh injections of cash.

It is surely wrong to say that Babbage formally asked the government to finance the building of the Analytical Engine. What he did undoubtedly feel was that, given his work on that engine, he was by 1842 in a position to design a better difference engine; a few years later he spent much effort in preparing detailed plans for what he called Difference Engine No. 2.

The first 20 pages of chapter 4 deal with the pioneering work in electronic computers done at the University of Pennsylvania in the mid-1940s. This led first to the ENIAC and then to the proposals on which the modern stored-program computer is based. The authors are clearly aware of sensitive areas where there have been differences of opinion and where feelings have sometimes run high, and they are evidently concerned to tread with care. I think they have been successful. The perceptive reader will come away with a picture that is well balanced in all essentials and with some feeling for the controversies.

This book is not a history of computer technology, nor is it a history of the ideas behind the technology. The authors' interest is in what may be called small-scale economic history: that is, the economic history of one particular industry. Much space is devoted to the ups and downs of companies, the manoeuvres of their senior managements, and their marketing policies. It would seem to have been a deliberate decision on the part of the authors to slant the book in this way. Yet from the beginning it has been technology that has driven the industry, and I wish that this fact could have been brought out more clearly.

The early computers were painfully lacking in reliability, speed and storage capacity, but they were such miracle-workers that scientific researchers were eager to exploit them. But it was obvious to everybody what needed to be done and there were plenty of able and innovative research engineers anxious to get on with the job. Progress was slow at first, but by the late 1960s the rate of technical progress on the hardware front was nicely matched to what the industry could handle. Great strides were made with both minicomputers and mainframes. The industrial leaders felt that they were firmly in control.

All went well until the late 1980s when progress in the semiconductor industry began to run away. More and more transistors could be put on a single silicon chip and, as the individual transistors were

smaller, it followed from the laws of physics that they ran faster. The result was that by 1986 it had become possible to build desktop computers that would out-perform the much larger, heavier and more expensive minicomputers and even begin to threaten mainframes. Overnight, the production facilities and organizational structures that had been built up for the manufacture of minicomputers became millstones around their owners' necks. The captains of industry, who had felt so confident, were still at their posts, but they were no longer in control of events. The painful consequences for the older companies in the industry are still in the memory of everybody concerned. Many companies collapsed while others, including the largest companies, underwent severe downsizing.

The book is intended neither for serious students of computer history nor for those interested in the history of ideas, although undoubtedly some of them will find certain sections of interest. As a popular book, however, it is to be welcomed, and I would expect it to do well. □

Maurice V. Wilkes is at Olivetti Research Ltd, 24a Trumpington Street, Cambridge CB2 1QA, UK.

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Spot the Difference — Babbage's engines bore little resemblance to the computers of today.

thing. The book ends with a chapter on the Internet and the World-Wide Web.

Inevitably, one judges a wide-ranging work such as this by the way it deals with topics of which one has specialist knowledge. I found that the treatment of the pioneering work between 1943 and 1946 at the University of Pennsylvania was good; I was much less happy with the section on Charles Babbage in chapter 3, although the comments about Lady Lovelace are well in line with the opinion of those best able to judge.

Babbage's manuscript notebooks are very far from being filled with "impenetrable scribbling" as the authors assert; in fact, Babbage's reputation today among interested scholars rests largely on the unpublished material they contain.

The first machine that Babbage proposed, the Difference Engine, was limited to the tabulation of polynomials, whereas the Analytical Engine that followed it would have been a genuine general-

Scientific ruralism

David Coleman

The Human Biology of the English Village. By G. Ainsworth Harrison. Oxford University Press: 1995. Pp. 147. £39.50.

FOR more than 15 years, from the mid-1960s, Geoffrey Ainsworth Harrison's department at the University of Oxford carried out the most innovative venture in biological anthropology in the United Kingdom: to assess and describe the human biology of the population of the Otmoor villages, a rural area near Oxford. Specialists know the papers. Now we have Otmoor the book, summarizing with much charm the key findings in some 150 readable pages.

The Otmoor project, begun in 1965, was comprehensive, multidisciplinary and ambitious. Its specific aims were to establish the relationship between human genetic structure, geographical mobility and mate selection, genetic components of health and well-being, and, above all, the nature and sources of variety in a modern population.

As a study area, Otmoor has the merit of being distinct and relatively isolated and having a separate identity in the eyes of its inhabitants and neighbours. The 17 villages in 10 ecclesiastical parishes date from Anglo-Saxon times. Otmoor has none of the ex-tremes of isolation that make some human biological studies so unrepresentative.

More than 30 papers are synthesized here, with topics grouped into seven chapters on population structure, social class, mobility and ancestry, biological variation, marriage and social mobility, and the biology of everyday life and health, with a final chapter on surnames by Gabriel Lasker, who has collaborated with the study over many years.

Otmoor's fairly complete parish records were scoured to provide trends in births, deaths and marriages and then, through record linkage, to reconstruct the life events of individuals. But because the crucial facts of population size are absent before 1801, except for fragments, the vital statistics remain numerators in search of a denominator. Nonetheless, estimates indicate that villages consisted of between 40 and 86 houses in the seventeenth century, and that population began and ended the period at around 2,500.

Population movements, past and present, have churned up and flushed out the population of Otmoor. Even in the eighteenth century, more than 35 per cent of spouses married outside their parishes, increasing to 80 per cent after the 1850s. The distance between them increased

from eight miles to thirty — a triumph of the railway and the bicycle over feet. The pattern of exchange reflects the geographical features of the area, local perception of 'otherness' and neighbourhood knowledge.

A matrix model shows the effects on ancestry of movement between parishes and between social classes. The model shows the number of generations required to generate an indistinguishable 95 per cent common ancestry between the populations, starting from an (imaginary) zero, and the pattern of relatedness that emerged. Without contributions from the 'outside world', it takes 50 generations to produce even 60 per cent common ancestry; with the outside world, just ten. A monolithic 'outside world' with such potent effects needs further subdivision.

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All is quiet in the English village of Islip. But scientists are at work in the Otmoor villages.

Such ingenious researchers could surely have produced some further structuring of this concept. Their migration matrix model also, of course, assumes stochastic behaviour by participants, another assumption in need of refinement.

These results forecast no genetic heterogeneity in the Otmoor populations, a proposition tested with data on seven blood groups, three red-cell enzymes and four serum proteins. There is little heterogeneity in polymorphic traits, but plenty in other interesting variables with strong environmental influence, notably IQ and stature, strongly correlated with migration status and with social class.

The chapters on the biology of everyday life and on health are the most path-breaking, relating physiology (adrenaline excretion) to environment, domestic life and kin, personality, occupation and lifestyle (but not genotype). Fascinating time-budget data surprisingly throw no light on sex or reproduction. Kinship is alive and well, more important for natives (80 per cent of local women see a relative in the past week, compared with 58 per cent of in-migrants). Only 23 per cent report an 'active social life'; 26 per cent have little or none.

An important innovation explores the relationship of sleep duration and delay (latency) — a good indicator of well-being

to psychological factors, occupation and so on. Boredom, frustration and tiredness enhance catecholamine excretion, but the relation of these hormones to health remains unclear. Housewives and clerical workers are less healthy than average (as indeed are women in general), plagued with 'too much time on their hands'. Busy people excrete less cortisol. But, frustratingly, too much pressure may also increase output. Psychologically related illness and incapacity emerge as a particularly coherent grouping.

Lasker's innovative measure, the coefficient of relatedness through isonymy, is population genetics without genes, estimating genetic relatedness of populations through the frequency of identical surnames; here on average 75×10^{-5} . The coefficient based on rarer surnames correlates well with relatedness calculated from marital migration; low levels underline the fact that local loyalties are environmental, not genetic.

In conclusion, there is no conclusion, although some of the chapters have concluding sections. The book is full of fascinating material. Because so much of the work is new, many of the lines that Harrison and his colleagues have begun cannot easily be related to other findings. For example, few comparative data on sleep and its correlates exist for 'natural' human populations, so Harrison and his colleagues may have been victims of their own innovation. Similarly, the relevance of the findings on 'stress' and catecholamine excretion remains difficult to evaluate without definitions and consequences being made clear. And although reproduction is a preoccupation of the historical section, it hardly features in the later analysis, where its correlates could have been most minutely examined.

'Scientific ruralism' peeps out from time to time; urban areas bad, rural ones good. This is axiomatic in British society, bolstered in biology by the notion that our palaeolithic genes equip us for life in Rose Cottage, not Railway Cuttings. But as the book shows, the Otmoor population, like most other rural populations, has been urbanized by migration, media and commuting. We are all townies now.

The Otmoor study and its successful completion are a great tribute to Harrison and his collaborators. Every researcher in human biology will want to read this admirable synthesis of their results. Chasing some of the numerous hares started from the Otmoor undergrowth will keep the profession busy for some time. □

David Coleman is in the Department of Applied Social Studies and Social Research, University of Oxford, Barnett House, Wellington Square, Oxford OX1 2ER, UK.

Cyclopia and defective axial patterning in mice lacking *Sonic hedgehog* gene function

Chin Chiang^{*†}, Ying Litingtung^{*†}, Eric Lee^{*}, Keith E. Young[†], Jeffrey L. Corden[†], Heiner Westphal^{*} & Philip A. Beachy[†]

^{*} Laboratory of Mammalian Genes and Development, National Institute of Health, Bethesda, Maryland 20892, USA

[†] Howard Hughes Medical Institute, Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA

Targeted gene disruption in the mouse shows that the *Sonic hedgehog* (*Shh*) gene plays a critical role in patterning of vertebrate embryonic tissues, including the brain and spinal cord, the axial skeleton and the limbs. Early defects are observed in the establishment or maintenance of midline structures, such as the notochord and the floorplate, and later defects include absence of distal limb structures, cyclopia, absence of ventral cell types within the neural tube, and absence of the spinal column and most of the ribs. Defects in all tissues extend beyond the normal sites of *Shh* transcription, confirming the proposed role of *Shh* proteins as an extracellular signal required for the tissue-organizing properties of several vertebrate patterning centres.

EXPERIMENTAL vertebrate embryology has demonstrated the capacity of certain tissues to influence the pattern of cell types within adjacent embryonic tissues, presumably through the action of secreted signalling molecules. Vertebrate gene products related to that of *hedgehog* (*hh*), first characterized as encoding a secreted protein signal in *Drosophila*^{1,2}, have emerged as the molecules likely to be responsible for several such patterning activities. The most-studied member of this multi-gene family in vertebrates is *Sonic hedgehog* (*Shh*), which has been isolated from many species, including mouse, chicken, rat, frog, fish and human³⁻⁹. These initial studies of *Shh* gene expression demonstrated an association with embryonic structures such as the notochord, the floorplate of the neural tube, and the posterior margin of tetrapod limb buds, all of which are known to have organizing properties in transplantation and grafting experiments.

The expression of *Shh* at these locations and the secretion of the *Shh* protein suggest that it is involved in patterning. Support for this suggestion comes from experimental expression or placement of the *Shh* protein at ectopic locations in embryos, and from treatment of explanted embryonic target tissues with purified protein. Thus, for example, cells infected with a retrovirus carrying the chicken *Shh* gene or transfected with a mouse *Shh* expression construct are capable of inducing mirror-image limb-digit duplications when grafted to the anterior margin of the embryonic chick limb bud^{4,7}; similar duplications resulted from grafting of beads impregnated with purified protein to the anterior limb bud margin¹⁰. In the neural tube, ectopic expression of *Shh* promotes the expression of genes normally expressed at or near the ventral midline, analogous to the effects of ectopic notochord grafts^{3,5,6,11}. These ventral patterning effects extend to the brain and eyes^{9,12-14} and to the somites, where *Shh* seems to promote formation of sclerotome ventrally and to repress more dorsal somite fates^{15,16}. Treatment of neural plate and presomitic mesoderm explants with purified protein also induced cell type-specific genes, with floorplate markers induced at high concentrations and motor neuron and sclerotome markers at lower concentrations¹⁶⁻¹⁸.

These experimental approaches demonstrated a potential role for *hh* activity in several aspects of embryonic patterning. However, because multiple vertebrate *hh* genes display similar signalling activities^{8,9,14}, these studies did not establish a unique requirement for *Shh*. To examine more directly the role of *Shh* in normal embryonic patterning and to search for other as-yet

unidentified patterning activities, we undertook the targeted mutagenesis of the *Shh* gene in the mouse.

Targeted disruption of *Shh*

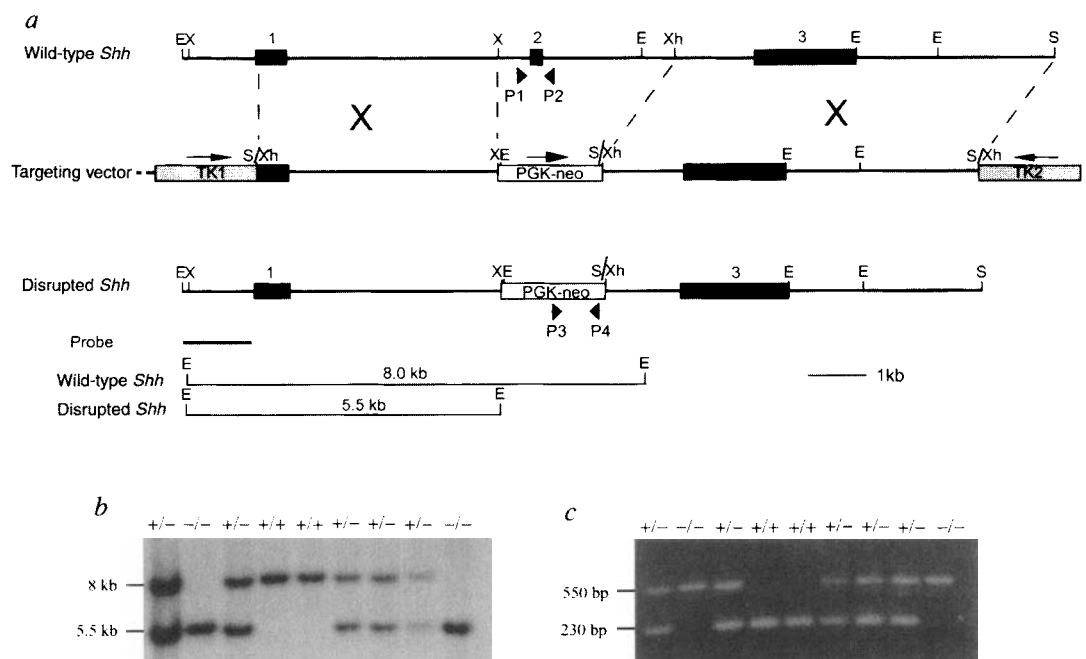
Mice homozygous for a disrupted *Shh* gene were generated by using homologous recombination in embryonic stem (ES) cells (Fig. 1a). In the targeting vector, a PGK-*neo* cassette replaced exon 2 and portions of the flanking introns, leaving coding sequences that would result in a truncated protein (Fig. 1a). This deletion causes a loss of 97 of the 198 residues within the N-terminal product of *Shh* autocatalytic processing, including most of the $\alpha + \beta$ sandwich (both of the α -helices and the central four of six β -strands) that forms the core of the N-terminal domain structure¹⁹. This domain is responsible for all *Shh* signalling activity, including the induction of a variety of ventral neural and somite cell types, and also the induction and patterning of distal limb structures^{9,10,12,17,18,20,21}. The targeting vector also contains two thymidine kinase genes at positions flanking the *Shh* genomic region for counter-selection of ES cells.

Southern analysis of 200 ES clones selected for thymidine kinase and neo resistance revealed 19 with a 5.5-kb *EcoRI* fragment, indicative of the deletion of exon 2 (data not shown). Chimaeric mice were generated by blastocyst injections from six independent ES cell lines containing the disrupted *Shh* allele. Two *Shh* mutant lines were then established through germline transmission of ES clones 199 and 184. Genotypes of the progeny were determined using Southern and polymerase chain reaction (PCR) analyses (Fig. 1b, c). Heterozygous mice seem normal, but only two homozygous mutant stillborn pups from more than a thousand progeny were recovered from crosses of *Shh*^{-/-} individuals. Genotypic analysis of embryos from 7.5 to 18.5 days post-coitum (d.p.c.) showed no obvious deviation from the expected Mendelian ratio (data not shown), indicating that most *Shh* mutant embryos die at or just before birth, with probable rapid cannibalization of stillborn pups.

Abnormal anatomy and histology

The earliest detectable morphological defects occur in the future forebrain region of homozygous mutant embryos at embryonic day 9.5 (E8.5). The cephalic neural tube at this stage has not yet closed, and the neural folds are quite extensive with well-defined lips that extend to a clearly defined ventral midline. In *Shh* mutant

FIG. 1 Disruption of the *Shh* gene by targeted recombination. **a**, Maps of the wild-type *Shh* locus, the targeting vector, and the disrupted *Shh* allele. Relative positions of the three *Shh* exons are indicated by black boxes. The map of the targeting vector shows the replacement of exon 2 and flanking genomic sequences by the Neomycin gene (*neo*), and the locations of two flanking HSV thymidine kinase genes (*TK*). Arrowheads denote the sites within the wild-type and the disrupted *Shh* alleles from which PCR primers are derived; lines at the bottom indicate expected sizes for *Eco*RI-generated fragments detected with a 5' flanking genomic probe from wild-type and disrupted *Shh* alleles. **b**, Southern analysis of DNA from individual embryos, the progeny of a heterozygous mating. Sizes of the wild-type and the targeted alleles are indicated by 8-kb and 5.5-kb hybridizing bands, respectively. **c**, PCR analysis of the same DNA samples as in **b**. The 230-bp (primers P1 and P2) and 550-bp (primers P3 and P4) amplification



products are specific to exon 2 of the wild-type *Shh* and to the *neo* gene within the disrupted *Shh* locus, respectively.

embryos, however, the midline is indistinct (compare Fig. 2a with Fig. 2b; arrow), the ventral lips of the cephalic folds are fused, and the normally separate optic vesicles appear instead as a continuous single vesicle protruding at the ventral midline. The cephalic defects in *Shh* mutant embryos become even more apparent when the neural tube closes, with an overall reduction in size of the brain and spinal cord. This is especially apparent at the mesencephalic/diencephalic junction of E9.5 embryos, where floor and roof of the neural tube are almost in contact with each other (arrowhead in Fig. 2c). By E11.5, as normal brain structures become more prominent, the small size and defects in the presumptive midbrain and forebrain of *Shh* mutant embryos can be clearly observed (Fig. 2d); the most obvious specific defect is the absence of externally visible lateral eye structures. By E15.5 (Fig. 2e), *Shh* mutant embryos exhibit severe growth retardation throughout most of the embryo and lack the distinct forelimb and hindlimb structures found in their normal littermates. In addition, relative growth deficits in the forebrain and in craniofacial structures are so extreme that normal facial features such as the eyes, nose and oral structures are not identifiable, and the sole remaining external feature of the mutant head is a proboscis-like extension that protrudes from the rostral midline. Gross inspection and preliminary histological analysis of the internal organs of E18.5 mutant embryos revealed abnormalities of heart, lung, kidney and foregut development (C.C. *et al.*, unpublished data), although asymmetry of early heart looping and internal organ situs appeared normal in all mutant embryos examined.

As noted above, bilateral eye structures are not observed in *Shh* mutant embryos at any stage of development, and the optic vesicle is single and continuous from its earliest appearance at ~E8.5. At E9.5, expression of the gene *microphthalmia* can be detected throughout this vesicle (Fig. 2f, g), suggesting that these cells are precursors of pigmented retinal epithelium²². Indeed, by E15.5 an accumulation of pigmented tissue is found consistently at the base of and just under the proboscis of these mutant embryos (Fig. 2e, arrowhead). In addition, expression of *Fgf-8* at E10.5 revealed a single nasal pit at the midline instead of the bilateral pits normally observed (compare Fig. 2h with Fig. 2i). Cross-sections of the neural tube at the level of the spinal cord and at other axial levels also revealed the absence of a morphologically distinct floorplate in E9.5 *Shh* mutants, with the ventral region of the neural tube

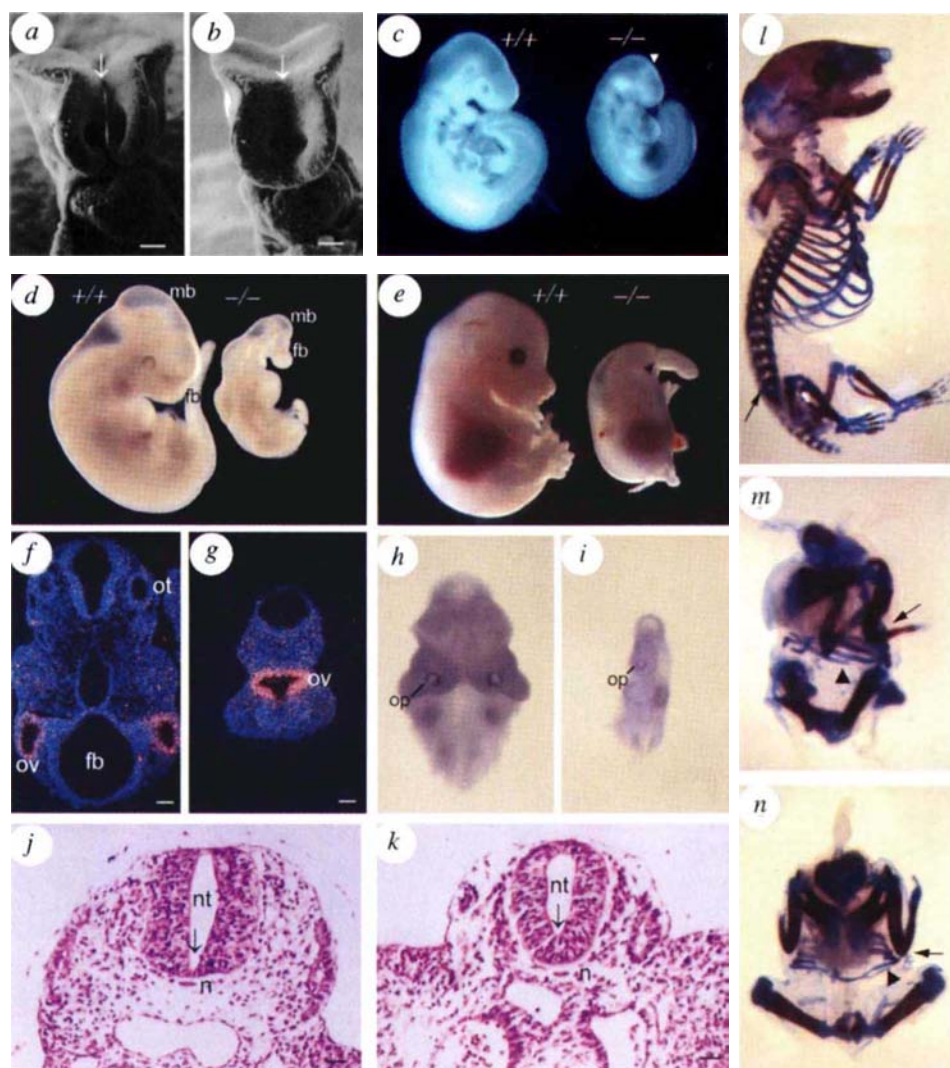
consisting instead of a thick epithelium (compare Fig. 2j with Fig. 2k; arrow). Notochord tissue is evident in cross-sections from caudal and some rostral axial levels in mutant embryos at this stage (see also Fig. 3).

Staining of bone and cartilage also revealed defects in the cranio-facial, axial and appendicular skeleton of *Shh* mutant embryos. Cranio-facial bones are severely affected and are almost entirely absent (Fig. 2m, n) despite the presence and nearly normal appearance of the branchial arches at E9.5 (Fig. 2c). Defects of the cranio-facial skeleton are commonly observed in association with neural tube defects, and in the case of *Shh* mutants these may be a secondary consequence of the extreme midbrain and forebrain defects. In the axial skeleton of *Shh* mutant embryos, most sclerotomal derivatives including the entire vertebral column are absent, with only five or six rib cartilages remaining. Other rib structures are difficult to assign in the absence of a vertebral column, but their cartilaginous composition suggests an anterior and ventral nature. In the appendages, distal structures are most affected, with a complete absence of digits and absence or fusion of the paired distal limb bones. Thus, in the hindlimb, which is more severely affected, the tibia and fibula are completely absent, although the femur is formed (Fig. 2m, n). In the forelimb, a bony extension of the humerus may represent either a fused ulna/radius or simply a long, bent humerus; these possibilities are difficult to distinguish given the absence of cartilage, which normally separates these bones at the elbow joint (Fig. 2m, n; arrow).

Defective axial structures

Because notochord tissue seems to be present in caudal but not rostral cross-sections of E9.5 *Shh* mutant embryos, we sought to determine whether the notochord is established normally or displays defects at earlier stages. Fate mapping and transplant studies have shown that the regressing node is the primary source of notochord tissue during early embryonic development^{23,24}. The *Brachyury* gene, which encodes a DNA-binding protein and is required for differentiation of the notochord, is normally expressed in the primitive streak and in the node and developing notochord of E7.5 and E8.5 embryos²⁵. In E7.75 *Shh* mutant embryos, *Brachyury* gene expression appears normal, consistent with the presence of the node at this early stage of development

FIG. 2 Abnormal anatomy and histology of *Shh* mutant embryos. *a–e*, External morphology of wild-type (+/+) and mutant (–/–) embryos. *a, b*, Scanning electron micrographs of the rostral ends of E8.5 wild-type (*a*) and mutant (*b*) embryos; note the lack of a distinct midline (arrow) and the single continuous optic vesicle at the ventral midline of the mutant embryo. *c*, Lateral view of E9.5 embryos. An abnormal cephalic flexure and the apposition of ventral and dorsal neural tube surfaces is indicated by the arrowhead. *d*, Lateral view of E11.5 embryos. Note the reduction in overall size, the abnormal forebrain morphology, and the lack of bilateral eye structures in the mutant embryo. *e*, Lateral view of E15.5 embryos. At this stage the craniofacial region of the mutant embryo consists of a long proboscis, at the base of which is an accumulation of pigmented cells (arrowhead). Note the stunted limbs of the mutant, *f, g*, Cross-sections of E9.5 wild-type (*f*) and *Shh*^{–/–} (*g*) embryos at the level of the optic vesicles hybridized with *microphthalmia* (*mi*) antisense RNA. Note the presence of a single *mi*-expressing optic vesicle in *Shh*^{–/–} embryos. *h, i*, Facial view of whole-mount *in situ* hybridization with *Fgf8* antisense RNA to E10.5 wild-type (*h*) and *Shh*^{–/–} (*i*) embryos. *Fgf8* expression highlights a single nasal pit forming within a single olfactory placode in the mutant embryo. *j, k*, Cross-sections at the level of the hind limb of wild-type (*j*) and *Shh*^{–/–} (*k*) stained with haematoxylin and eosin. Note the lack of a floorplate in the ventral midline of the mutant neural tube (arrow), despite the apparent presence of notochord tissue. *l–n*, Bone (brown) and cartilage (blue) stains of E18.5 wild-type (*l*) and *Shh*^{–/–} embryos (*m, n*). In *Shh*^{–/–} embryos, most of the sclerotomal derivatives such as spinal column (*l*, arrow) are missing with the exception of some rib cartilages (*m, n*, arrowhead). The distal limb structures are also missing; note the absence of cartilage at the elbow joint in the mutant (*m, n*, arrow). Abbreviations: fb, forebrain; mb, midbrain; n, notochord; nt, neural tube; op, olfactory placode; ov, optic vesicle;



ot, otic vesicle. Scale bar, 100 μ m. For comparison, mutant embryos in *m* and *n* are shown at 2.5-fold magnification relative to the wild-type in *l*.

(data not shown). By E8.5, however, *Brachyury* gene expression becomes discontinuous in the rostral portion of mutant embryos (Fig. 3*a*). By E9.5, the rostral-most expression of the *Brachyury* gene is completely lost (Fig. 3*b*); this loss progresses in a rostral-to-caudal direction, as indicated by patchy *Brachyury* gene expression more caudally. The progressive, rostral-to-caudal loss of notochord tissue concomitant with loss of *Brachyury* gene expression suggests that *Shh* is required for the maintenance, but not the formation, of the notochord.

The *HNF-3 β* gene, which encodes a winged-helix transcription factor required for development of the axial structures^{26,27}, is expressed in the node, developing notochord, head process, floorplate and gut endoderm, in a spatial pattern that is similar to but slightly precedes that of *Shh*⁵. At E7.75, although the localization of *HNF-3 β* expression seems normal in *Shh* mutant embryos, the level of expression is consistently reduced in the notochord and in the head process (Fig. 3*c*). By E8.5, *HNF-3 β* expression in axial mesoderm is completely absent (Fig. 3*d*), although gut expression persists for at least one more day of embryonic development (data not shown); expression of *HNF-3 β* in the ventral neural tube of mutant embryos is never initiated. Initiation of *HNF-3 β* expression in the ventral neural tube in normal embryos occurs before the stage at which the notochord degenerates in *Shh* mutant embryos; the failure to initiate *HNF-3 β* expression in mutant neural tube thus suggests that *Shh* protein is the notochord-derived signal required for *HNF-3 β* induction in the normal neural tube.

The notochord is also known to induce differentiation of other ventral cell types within the neural tube, including motor neurons²⁸. The *Isl-1* gene (*Isl-1*) encodes a LIM homeodomain protein that is expressed in motor neurons, and mice lacking function of this gene fail to form motor neurons²⁹. We therefore examined mutant embryos for the expression of *Isl-1* protein as a marker for the presence of motor neurons. Normal expression of *Isl-1* in the neural tube is first detected at E9.5 in a punctate pattern of nuclear staining lateral to the floorplate (Fig. 3*e*). In *Shh* mutant embryos, *Isl-1* expression is absent in the neural tube at all axial levels (Fig. 3*f*), including caudal levels where the notochord persists. Failure to induce the *Isl-1* marker even in the presence of the notochord suggests that *Shh* protein from the notochord may be required for differentiation of motor neurons. Alternatively, differentiation of motor neurons may require signalling from the floorplate, or from both the notochord and the floorplate.

Dorsoventral pattern defects in the neural tube

Given the degeneration of the notochord and failure of the floorplate to develop in *Shh* mutant embryos, we examined dorsoventral (D–V) patterning of the neural tube at E11.5 by assaying the expression patterns of various *Pax* gene family members (reviewed in ref. 30). In the neural tube at the level of the prospective spinal cord, *Pax-3* is normally expressed in the alar plate, corresponding to the dorsal half of the neural tube (Fig. 4*a*).

Pax-6, in contrast, is expressed only weakly in the alar plate and more strongly throughout the basal plate except at the ventral midline (Fig. 4c). In *Shh* mutant embryos, high levels of *Pax-3* expression extend ventrally and across the midline where floorplate would normally be present (Fig. 4b). Similarly, expression of *Pax-6* also extends across the ventral midline in mutant embryos and is reduced to a level more characteristic of normal alar plate expression (Fig. 4d). In *Shh* mutant embryos, gene expression patterns in the ventral neural tube thus appear to lose the characteristics of normal basal plate (exclusion of *Pax-3*) and floorplate (exclusion of *Pax-6* from the midline) and acquire alar plate characteristics such as a high level of *Pax-3* expression and a reduced but uniform overall level of *Pax-6* expression. *Pax-2* expression, normally restricted to interneuronal regions of the alar and basal plates that lie adjacent to the sulcus limitans, also expands across the ventral midline in mutant embryos (Fig. 4e, f). The normal exclusion of *Pax-2* from dorsal parts of the neural tube is preserved, however, suggesting that dorsal pattern is maintained within *Shh* mutant embryos.

Because the notochord degenerates in *Shh* mutant embryos, the abnormal patterning of the ventral neural tube may be an indirect consequence of this loss rather than a direct consequence of loss of *Shh* function. To assess this possibility, expression of *Pax-3* within the E9.5 neural tube was examined in the caudal portion of mutant embryos where some notochord tissue is still present (compare Fig. 5a with Fig. 5b). The inability of notochord lacking *Shh* function to exclude *Pax-3* expression from the ventral portion of the neural tube, in conjunction with the results of ectopic expression^{3,5,6,9,11–13} and explant studies^{6,17,18,21}, suggests a direct role for *Shh* in neural tube patterning.

Patterning of paraxial mesoderm

Differentiation of the somite into dorsal dermatome, medial myotome and ventral sclerotome is mediated by inductive signals derived from surrounding tissues³¹. Previous studies have shown that *Shh* is capable of inducing expression of the ventral sclerotomal marker *Pax-1* and of suppressing the dorsal dermomyotomal marker *Pax-3*, either when expressed ectopically in the chick or when applied to presomitic mesoderm explants^{15,16,20}. The expression of *Pax-3* normally is confined to the dermomyotome of the differentiating somite (Fig. 5a). In E9.5 mutant embryos *Pax-3* expression is ventrally expanded (Fig. 5b), suggesting that the sclerotome may also be affected. Surprisingly, we observed weak but reproducible *Pax-1* expression in E9.5 mutant somites (compare Fig. 5c with Fig. 5d), with a region of higher expression in a block of five or six somites (compare Fig. 5e with Fig. 5f). By E11.5 all somite expression of *Pax-1* is lost (Fig. 5g, h), indicating that *Shh* is essential for the maintenance, but not the initiation, of sclerotomal *Pax-1* expression.

The presence of skeletal muscle in later-stage *Shh* mutant embryos (data not shown) indicates that differentiation of myotomal derivatives does not require *Shh* function. However, at least two myotomal components have been resolved within the somite, the medial *myf-5*-expressing component and the lateral MyoD-expressing component (reviewed in ref. 32). We observed significantly reduced accumulation of *myf-5* transcripts at E9.5 (Fig. 5i, j) and normal levels of MyoD protein at E10.5 (Fig. 5k, l) throughout the axis of mutant embryos. These results are consistent with reports that medial but not lateral myotomal components are dependent on axial structures^{33,34}, and further suggests the possibility that *Shh* function contributes to this signal. As also observed for the *Pax-1* sclerotomal marker, however, *Shh* function appears not to initiate but to enhance or maintain expression of the *myf-5* lateral myotomal markers.

Patterning of brain and eye

In comparison with their normal littermates, the brains of *Shh*^{-/-} embryos are reduced in size, with progressively more severe morphological defects observed in the hindbrain, midbrain and forebrain. To characterize these abnormalities further we exam-

ined the expression in *Shh* mutant embryos of several homeobox genes that are expressed in restricted regions of normal E11.5 brain. In the normal forebrain, *Emx-1* expression³⁵ is restricted to the dorsal telencephalon corresponding to the neocortex (Fig. 6a). In *Shh* mutant embryos, *Emx-1* expression can be detected throughout a single vesicle present in the midline (Fig. 6d), suggesting that the normally bilateral lobes of the telencephalon are fused to form a single midline structure and that ventral forebrain structures are lost. Consistent with this interpretation, expression of the *Otx-2* gene³⁶ is also lost in ventral domains of the telencephalon and in the diencephalon (compare Fig. 6b with Fig. 6e); indeed, most of the diencephalon as an identifiable structure is absent in *Shh* mutant embryos. Reduced expression of *Otx-2* in the mesencephalon is also consistent with the reduction in size and the abnormal morphology of the midbrain in the *Shh* mutant (Fig. 6e). The deficit in the midbrain would appear to be in the anterior, as most of the remaining midbrain cells express the *En-2* gene³⁷, normally restricted to the posterior midbrain (compare Fig. 6c with Fig. 6f). Expression of the *En-2* gene at the isthmus is essentially normal, consistent with the presence of the midbrain/hindbrain constriction and also with the normal appearance of *Pax-2* expression at this constriction (data not shown). The expression in normal anterior/posterior sequence of *Emx-1*, *Otx-2* and *En-2* in prospective forebrain, midbrain and hindbrain domains suggests that the major requirement for *Shh* gene function is for ventral cell fate specification rather than anteroposterior patterning.

The vertebrate eye originates from evagination of the optic vesicles from the lateral walls of the forebrain (reviewed in ref. 38). After the optic vesicles have formed they undergo an additional invagination to form double-layered cups that differentiate into a neural retina and a pigmented epithelium; these cups remain connected to the diencephalon by the optic stalks. In *Shh* mutant embryos the optic vesicles are fused at the midline and the optic stalks are deficient or absent. There is no invagination to form the characteristic double-layered optic cups, and the fused eye tissue at the midline forms a pigmented epithelium with no apparent differentiation of retinal tissue (Fig. 2f, g). To analyse eye patterning defects in mutant embryos further we examined the expression patterns of *Pax-6*, *Otx-2* and *Pax-2* genes in the E11.5 mutant eye. *Pax-2* and *Pax-6* have been shown to play important roles during eye development (reviewed in ref. 38) and serve as early markers for proximal cells that contribute to the future optic stalk, and for distal cells that contribute to both layers of the optic cup^{9,13}, respectively. The expression of *Pax-6* throughout most of the normal optic cup, including the pigmented epithelium and the neural retina, is shown in Fig. 6g. *Pax-6* expression occurs throughout the cyclopic mutant eye structure (Fig. 6j), which is constituted solely of pigmented epithelium. *Otx-2* expression similarly persists in the cyclopic *Shh* mutant eye (Fig. 6h, k). The expression of *Pax-2*, normally restricted to the optic stalks connecting the eye primordia to the brain (Fig. 6i), is absent in *Shh* mutant embryos (Fig. 6l), consistent with the histologically apparent absence of the optic stalk and the loss of ventral forebrain structures.

Discussion

Roles of *Shh* expression in neural patterning and notochord maintenance. One of the early functions of the notochord is to induce differentiation of ventral cell types such as floorplate cells and motor neurons in the overlying neural ectoderm. Our studies demonstrate, however, that, despite the presence of a differentiated notochord during early stages of development, floorplate cells and motor neurons are not induced in *Shh* mutants. Conversely, in other vertebrate mutants, such as the zebrafish *floating heat* (*flh*) mutant³⁹, or the murine *Brachyury* (T) mutant²⁵ and its zebrafish homologue *no tail* (*ntl*)⁴⁰, floorplate cells develop in rostral portions of the embryo despite the absence of a differentiated notochord. These notochord mutants, however, express *Shh* in presumptive notochord precursor cells in the midline. Expression of *Shh* in the absence of definitive notochord

differentiation may therefore be sufficient for ventral neural patterning, whereas notochord differentiation in the absence of *Shh* function is not. Together with the results of neural plate explant studies^{6,17,18,21}, these results suggest that ventral patterning by the *Shh* signal can operate independently of notochord development.

Our finding that *Shh* is not essential for node formation or for notochord differentiation is not surprising, as expression of *Shh* in the node is first detectable at the late streak to early head-fold stages, by which time a morphologically recognizable node and notochord are already present. The requirement for *Shh* in maintenance of the notochord, however, represents a previously

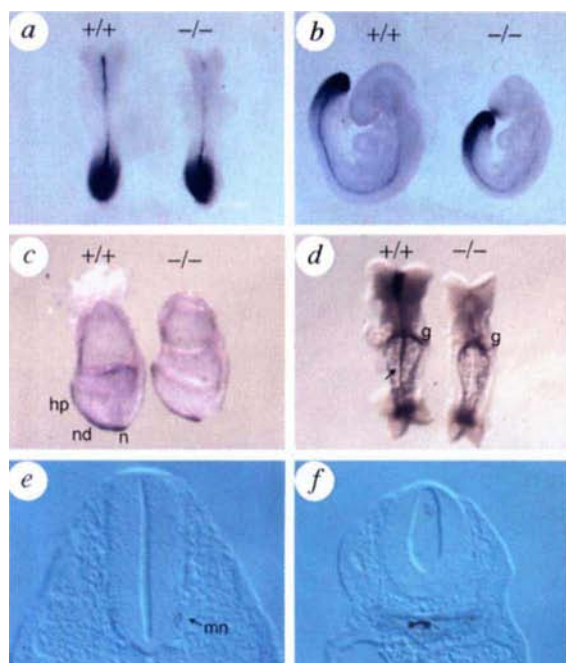


FIG. 3 Defective axial structures in *Shh* mutant embryos. *a, b*, Expression of *Brachyury* in E8.5 (*a*) and E9.5 (*b*) embryos. Note the discontinuity and loss of *Brachyury* expression in the rostral part of *Shh* mutant ($-/-$) embryos. *c, d*, Expression of HNF-3 β expression in the node (*n*), notochord (*nd*) and head process (*hp*) of *Shh* $^{-/-}$ embryos is similar to the normal pattern, although the level of expression is reduced. At E8.5 (*d*, ventral view), HNF-3 β expression in the floorplate and the notochord (arrow) is completely absent, although expression persists in the gut (*g*). *e, f*, Cross-sections through E9.5 wild-type (*e*) and *Shh* $^{-/-}$ (*f*) embryos at the level of the forelimbs labelled with Islet-1 antibody. Accumulation of Islet-1 protein in the motor neurons (*mn*, arrow) can be detected as bilateral punctate staining within the ventrolateral portion of the normal neural tube (*e*). This staining is completely absent in *Shh* $^{-/-}$ embryos (*f*).

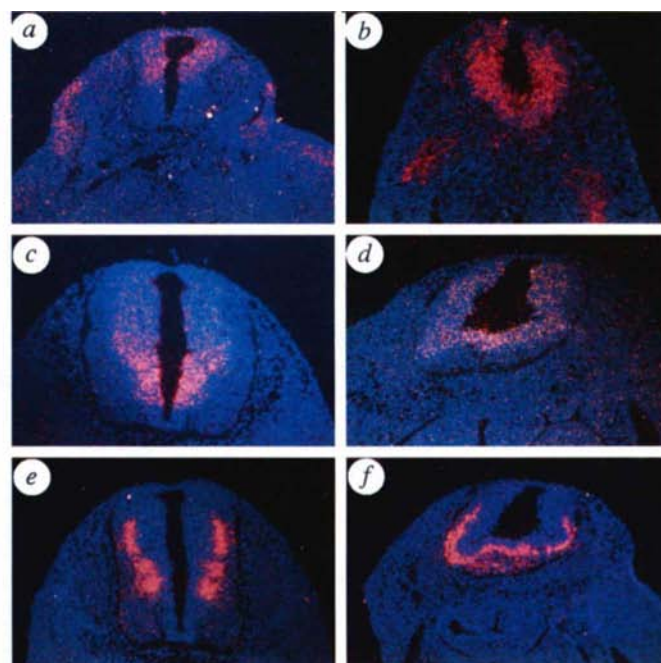
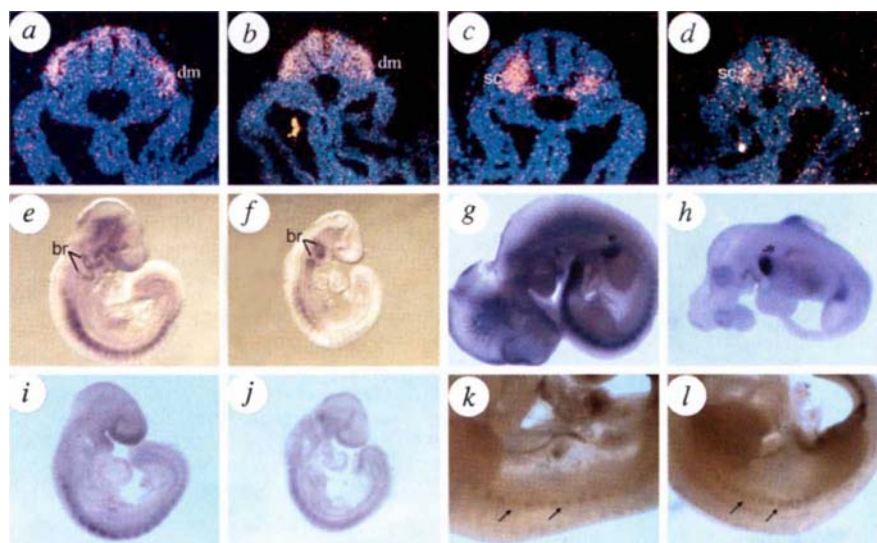


FIG. 4 Dorsoventral patterning defects in the neural tube of *Shh* mutant embryos. *a–f*, Cross-sections through E11.5 wild-type (*a, c, e*) and *Shh* $^{-/-}$ (*b, d, f*) embryos hybridized with ³³P-labelled antisense RNAs. *a, b* *Pax-3* is normally expressed in the alar plate of the neural tube (*a*) but in *Shh* $^{-/-}$ (*b*) expression expands across the ventral midline of the neural tube. *c, d*, *Pax-6* expression, normally high in the basal plate and weak in the alar plate (*c*), is distributed uniformly throughout the neural tube including the ventral midline at a reduced level characteristic of normal alar plate expression (*d*). *e, f*, Normal *Pax-2* expression is restricted to the interneuron region flanking the sulcus limitans (*e*) but in *Shh* $^{-/-}$ mutants it expands across the ventral midline of the neural tube (*f*). Note the exclusion of *Pax-2* expression from the dorsal midline in both wild-type and mutant embryos. Scale bar, 100 μ m.

FIG. 5 Somite development in *Shh* $^{-/-}$ embryos. *a–d*, Cross-sections of wild-type (*a, c*) and *Shh* $^{-/-}$ (*b, d*) E9.5 embryos showing somite expression of *Pax-3* (*a, b*) and *Pax-1* (*c, d*). Normal expression of *Pax-3* in the dermomyotome (*dm*) is ventrally expanded in *Shh* $^{-/-}$ embryos and *Pax-1* expression is reduced. *e–h*, Whole-mount E9.5 (*e, f*) and E11.5 (*g, h*) embryos showing *Pax-1* expression in wild-type (*e, g*) and *Shh* $^{-/-}$ (*f, h*) mutant embryos. Relative to wild type, the reduced level of *Pax-1* sclerotome expression in the E9.5 mutant (*f*) is completely lost by E11.5 (*h*), whereas forelimb expression is largely normal (asterisks). *i, j*, Whole-mount E9.5 wild-type (*i*) and *Shh* $^{-/-}$ (*j*) embryos showing expression of *myf-5* RNA. *k, l*, E10.5 wild-type (*k*) and *Shh* $^{-/-}$ (*l*) embryos showing MyoD protein accumulation in the myotome (arrows). MyoD protein expression in *Shh* $^{-/-}$ mutant embryos is similar to that in wild type, but expression of *myf-5* RNA in mutant embryos is significantly reduced. Abbreviations: br, branchial arches; dm, dermomyotome; sc, sclerotome.



unforeseen role. This maintenance function may represent an autocrine action of the *Shh* signal produced within the notochord. Alternatively, notochord maintenance may require signals derived from other tissues, such as the floorplate, whose formation is dependent on earlier *Shh* function; this possibility seems unlikely, however, given the persistence of notochord in *cyclops* mutants of the zebrafish, which lack floorplate cells⁴¹, and in chick embryos from which the neural tube has been extirpated before floorplate formation³³.

Regulatory interactions in axial tissues. Analysis of the *Shh* mutant phenotype revealed several features of the regulatory interactions between genes expressed in axial tissues. Previous studies had suggested the existence of a positive feedback between the *Shh* signal and the transcription factor *HNF-3 β* for mutual maintenance of expression in the neural tube. We have found here that *Shh* is indeed required for the later *HNF-3 β* expression, but our analysis also shows that early expression of *HNF-3 β* in the axial mesoderm of late streak to early head-fold stages is initiated independently of *Shh* function. Regulation of *HNF-3 β* expression thus changes from early independence to a later requirement for *Shh* function.

Another example of changing regulatory relationships in axial structures is the shift of *Brachyury* gene expression from early dependence on *HNF-3 β* function^{26,27} to later independence, as indicated by our observation in the *Shh* mutant that *Brachyury* gene expression persists considerably longer than expression of *HNF-3 β* . Previous studies have suggested that the maintenance of *Brachyury* expression in the notochord is mediated through autoregulation⁴². Alternatively, maintenance of *Brachyury* expression could involve mouse homologues of genes such as *flh*, whose function is required for *ntl* expression in zebrafish³⁹.

The developmental logic underlying these dynamically changing regulatory relationship seems to be an early marshalling of axial gene expression under the regulatory control of the *HNF-3 β* transcription factor (or perhaps other transcription factors acting upstream). Once expression of axial genes is established, *Shh* and *HNF-3 β* expression become mutually interdependent and expression of both genes is established in the ventral midline of the neural tube through the signalling action of the *Shh* protein. Restriction of other early axial genes such as *Brachyury* to expression in the notochord is accomplished by uncoupling expression from *HNF-3 β* regulatory input, and possibly by an increasing reliance on auto-regulation or regulation by other notochord-specific genes such as *flh*.

The role of *Shh* in development of the axial skeleton. *Shh* protein has been implicated as the notochord-derived signal responsible for induction of the sclerotome^{15,16,20}, and the reduced level of *Pax-1* expression and the absence of most sclerotomal derivatives in *Shh*^{-/-} embryos suggest a role for *Shh*. Furthermore, the observed skeletal defects in *Shh*^{-/-} embryos overlap with those of *Pax-1* null embryos, in which ventral portions of the vertebrae, such as vertebral

bodies, intervertebral discs and a few medial portions of the ribs, are missing⁴³. Nevertheless, some expression of *Pax-1* occurs in the absence of *Shh* function, indicating that *Shh* is not absolutely required *in vivo* for specification of sclerotomal cell fates. The role of *Shh* thus might be to maintain or expand the population of committed sclerotome cells. Consistent with this view is the observation that *Shh* protein is a potent mitogen for presomitic explants²⁰, and that low levels of *Pax-1* are expressed in these explants even in the absence of induction by notochord or *Shh* protein¹⁶. The absence of the entire vertebral column in *Shh*^{-/-} embryos, as opposed to a partial loss in *Pax-1* mutant embryos, suggests that sclerotome-promoting effects of *Shh* may involve modulation of other genes in addition to *Pax-1*; one such gene could be *Pax-9*, which is expressed in the sclerotome and has an expression pattern similar to that of *Pax-1* (ref. 44).

The role of *Shh* in limb development. Based on the initial view of *Shh* and the zone of polarizing activity in anteroposterior limb patterning, an early prediction might have been that loss of *Shh* function would lead simply to anterior/posterior mispatterning of

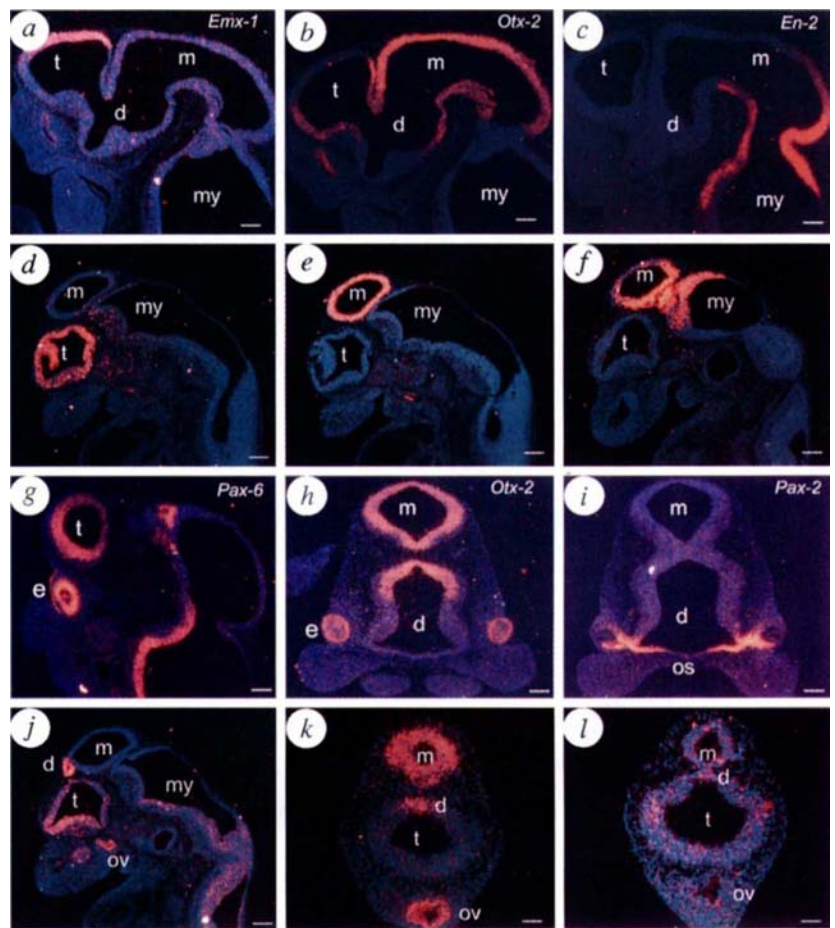


FIG. 6 Brain and eye patterning defects in *Shh* mutant embryos. *a–f*, Parasagittal sections of E11.5 wild-type (*a–c*) and *Shh*^{-/-} (*d–f*) embryos. *a, d*, Expression of *Emx-1* is normally restricted to the dorsal telencephalon (*a*). In the *Shh*^{-/-} embryo, *Emx-1* expression can be detected throughout a single telencephalic vesicle located at the midline (*d*). *b, e*, Normal expression of *Otx-2* in the ventral telencephalon (*b*) is absent in *Shh*^{-/-} embryos (*e*). *c, f*, *En-2* expression marks the midbrain/hindbrain boundary, which is present in *Shh*^{-/-} embryos. *g–l*, Parasagittal (*g, j*) and coronal (*h, i, k, l*) sections through the eye of E11.5 wild-type (*g–i*) and *Shh*^{-/-} (*j–l*) embryos. *g, j*, *Pax-6* expression is retained in the single optic vesicle present in *Shh*^{-/-} embryos. *Pax-6* is also expressed in the remnant of the dorsal diencephalon of *Shh*^{-/-} embryos. *h, k*, *Otx-2* is expressed in the midbrain, the remnant of the dorsal diencephalon, and the single optic vesicle of *Shh*^{-/-} embryos. Arrow indicates the floorplate in the normal mesencephalon, which is absent in *Shh*^{-/-} embryos. *i, l*, The optic stalks and *Pax-2* expression are absent in *Shh*^{-/-} embryos. Abbreviations: d, diencephalon; e, eye; m, mesencephalon; my, myelencephalon; ov, optic vesicle; os, optic stalks; t, telencephalon. Scale bar in *a–j*, 100 μ m; in *k, l*, 35 μ m.

the limbs⁴. The dramatic loss of distal structures observed here, however, indicates that *Shh* plays an important role in promoting distal limb fates. Consistent with such a role for *Shh*, experimental amputations of posterior portions of chick limb buds produced dramatic losses of distal structures⁴⁵. These distal truncations resemble those observed in *Shh* mutant mice, and are far more severe than defects caused by anterior amputations of comparable extent. One possible link between the distal truncations caused by posterior limb-bud amputation and loss of *Shh* function is the apparent role for fibroblast growth factor (FGF) signals in limb outgrowth and the requirement for *Shh* in maintenance of FGF-4 expression in the posterior apical ectodermal ridge⁴⁶ (C.C. *et al.*, manuscript in preparation). Normal expression of *Shh* at the posterior margin of the limb bud is thus implicated in dynamically shaping limb pattern during outgrowth, presumably through a fairly local induction of cell proliferation, with distal structures deriving largely from the postaxial region of the limb bud⁴⁷.

The role of *Shh* in establishing the ventral midline of the brain and spinal cord. Throughout the neural tube, loss of *Shh* function results in failure to establish cell fates and gene expression patterns characteristic of the ventral midline. Perhaps the most striking defects in *Shh* mutant embryos occur in the forebrain and its derivatives, which at E11.5 consist of a single fused telencephalic vesicle that overlies a single fused optic vesicle situated at the base of an externally apparent proboscis. This phenotype is reminiscent of a spectrum of human congenital malformations referred to collectively as holoprosencephaly, which in its most severe form is accompanied by cyclopia, a single nasal chamber, a single telencephalic vesicle, and other forebrain and facial midline defects⁴⁸. An extensive review of cyclopia and associated developmental malformations raised the key question of whether the eye anlage in the early rostral neural plate is normally single and continuous or instead is bilaterally divided^{49,50}, and concluded that the early eye field is continuous across the midline and that later subdivision of this single eye field at the midline depends on influences from prechordal plate mesoderm. These conclusions were based on elegant experiments involving *in vivo* culture of median or lateral strips from rostral neural plate, either alone or recombined with underlying prechordal mesoderm or prechordal plate mesoderm. Also consistent with these conclusions is the induction of cyclopia by the removal of prechordal plate mesoderm from beneath the early neural plate of amphibian embryos (reviewed in refs 49, 50).

Our study is consistent with the idea that *Shh* expression in prechordal plate mesoderm contributes to the signal that estab-

lishes the ventral midline of the brain and subdivides the eye field. The expression of *Shh* is normally first detected in the prechordal mesoderm underlying the rostral neural plate of mouse embryos at the midstreak stage^{3,7}. In the early head-fold stage of *Shh*^{-/-} embryos, *HNF-3 β* and *Brachyury* expression appear normal, indicating that the prechordal plate mesoderm is present during early embryogenesis. Rostral neural plate defects are morphologically apparent shortly thereafter, suggesting that, as observed for spinal cord regionalization and morphogenesis, it is the absence of the *Shh* signal rather than absence of axial mesoderm that is responsible for patterning defects. With regard to eye patterning, we have demonstrated that *Shh* is required for *Pax-2* expression and for optic stalk formation in the forebrain. These results are consistent with previous evidence from zebrafish studies suggesting that *hh* activity from the ventral midline normally stimulates expression of *Pax-2* in the adjacent optic stalk precursors and represses expression of *Pax-6*, thus restricting *Pax-6* expression and its associated retinal and epithelial fates to distal portions of the optic vesicle^{9,13}. These effects of *Shh* signalling identify a molecular pathway by which bilateral subdivision of the eye field at the midline might be accomplished while simultaneously imposing a proximo-distal patterning influence on the developing optic vesicle. □

Methods

Targeted recombination of the *Shh* gene. The targeting vector was constructed by inserting a 14-kb *SalI* (*Sal* sites were vector-derived) fragment of *Shh* genomic DNA into the *XhoI* site of a thymidine kinase-containing vector. To delete the *Shh* exon 2 region, the 3.0-kb *XbaI/XhoI* fragment was replaced with the *XbaI/SalI* fragment containing the P_{gk}-neo cassette. The final targeting vector was linearized at a unique *XhoI* site before use in the transfection of ES cells. R1 ES cells were grown, transfected and subjected to double selection^{26,51}. Homologous recombinant ES clones were identified by Southern analysis and further analysed using PCR primers specific to *Shh* exon 2 and to the *neo* gene to confirm the absence of exon 2 sequences in the disrupted gene. Frozen cells were expanded and used for injection into C57Bl/6J blastocysts. The primers were: P1, 5'-GACCATGTCTGCACACTAGGTTCC-3'; P2, 5'-GAAGGCC AGGAGGAGAAGGCTCAC-3'; P3, 5'-CTGTGCTCGACGTTGTCTACTG-3'; P4, 5'-GATCCCTCAGAAGAACTCGT-3'.

Histology and *in situ* detection of RNA and protein expression. Embryos were fixed in 4% paraformaldehyde overnight at 4 °C, dehydrated, embedded in paraplast, and sectioned at 5 μ m. Sections were stained with haematoxylin and eosin for histological analysis. Cartilage and bones were stained with alcian blue and alizarin red⁴³. *In situ* hybridization to tissue sections⁷ and *in situ* hybridization to whole embryos were performed²⁶. Whole-mount immunohistochemistry using Isl-1 antibody was performed essentially as described²⁹. After immunostaining, embryos were sectioned at 5 μ m.

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CORRESPONDENCE should be addressed to C.C. (e-mail: chiangc@dir6.nichd.nih.gov) or P.A.B. (e-mail: phil.beach@qmail.bs.jhu.edu), and requests for materials to H.W. (e-mail: hw@helix.nih.gov) or P.A.B.

Observations of stellar proper motions near the Galactic Centre

A. Eckart & R. Genzel

Max-Planck Institut für extraterrestrische Physik, D85740 Garching, Postfach 1603, Germany

EVIDENCE for a massive black hole at the centre of our Galaxy has been accumulating for the past two decades¹⁻⁷. Estimates of the mass of this region have hitherto been based on the spectroscopic determination of radial velocities for stars and gas near the Galactic Centre, combined with the assumption that the stars are moving on largely circular, isotropically distributed orbits. But if this assumption is incorrect, the observations can be explained with a much smaller central mass, perhaps obviating the need for a massive black hole. Here we report the proper motions (motions in the plane of the sky) of 39 stars located between 0.04 and 0.4 pc from the Galactic Centre. We find that the velocity dispersion estimated from the proper motions is in excellent agreement with that obtained from the radial velocities, indicating that the average velocity field is close to isotropic.

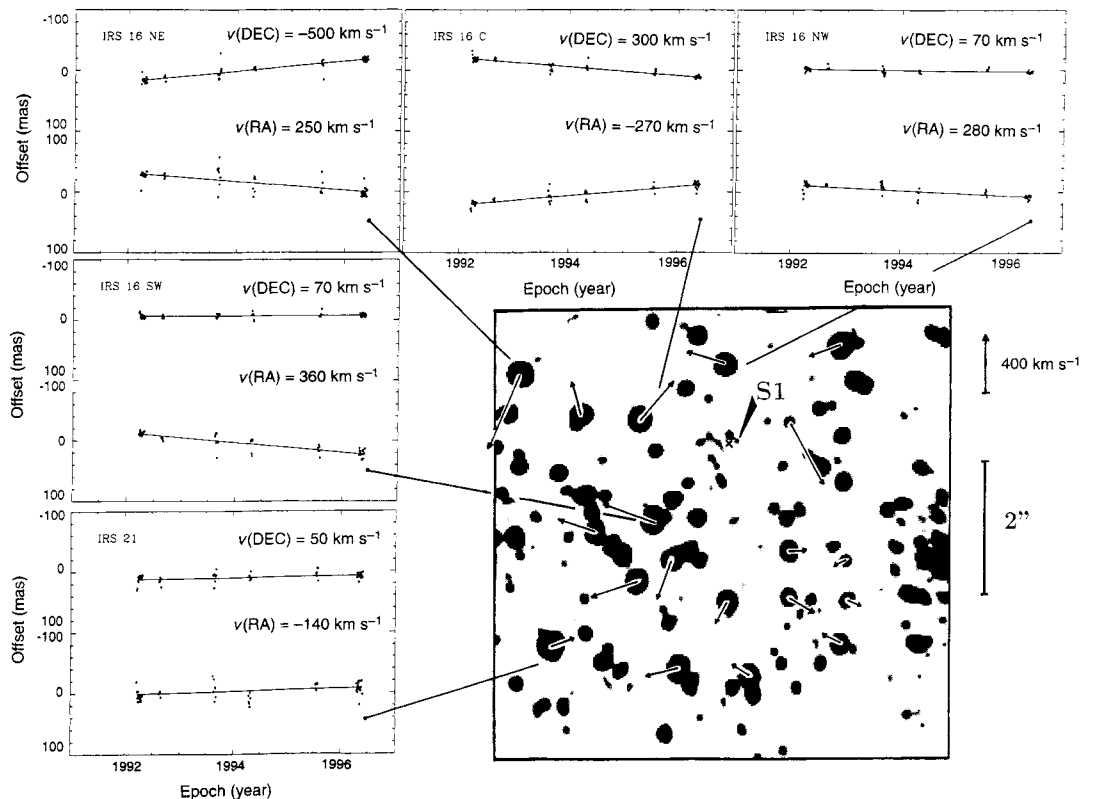
Taken together, the observations provide strong evidence for a central dark mass of $2.45 \pm 0.4 \times 10^6$ solar masses ($M_\odot$) located within 0.015 pc of the compact radio source Sgr A*. We place a lower limit of $6.5 \times 10^9 M_\odot \text{pc}^{-3}$ on the density of the central region, suggesting that it is most probably occupied by a massive black hole.

For determining the distribution of total mass from stellar dynamics without the *a priori* assumption of isotropic motions it is necessary to determine both the radial and proper motions of the stars. For this purpose we have been carrying out since 1991 a programme of high resolution 2.2- μm (K band) imaging at the 3.5-m New Technology Telescope (NTT) of the European Southern Observatory (ESO) in La Silla, Chile. Using the SHARP camera developed specifically for this project⁸, we have applied speckle imaging techniques to obtain diffraction-limited resolution (0.15 arcsec full-width at half-maximum, FWHM, at 2.2 μm ; see refs 9-12 and Fig. 1 legend for technical details). Further details of the proper motion analysis are given elsewhere¹³.

In the present analysis we have determined proper motions for 39 stars between 0.1 and 8.8 arcsec from Sgr A*. Table 1 lists the derived K-band magnitudes, offsets in terms of right ascension (RA) and declination (dec.) from Sgr A* (epoch 1994.27) and velocities, and (wherever available⁷) the radial velocities of these stars, together with their identification. As an example of the quality of the data we have obtained, Fig. 1 shows the RA and dec. offsets of five selected bright and isolated stars as a function of

FIG. 1 Proper motions of selected stars. The five insets show the changes of RA and dec. positions as a function of time, along with the best-fit proper motions. Offsets are relative to the positions determined at the base epoch (1994.27). Positions of the different data sets in each epoch are shown as black dots. Fit errors for positions in each data set and the dispersion of fitted positions in an epoch are very similar, indicating largely statistical errors. The locations of the five stars and the direction and size of their proper-motion vectors (plus others from Table 1) are shown in the adjacent grey-scale 2- μm image (0.15 arcsec FWHM; ref. 11). The cross denotes the position of Sgr A* (ref. 11 and K. Menten, A.E., M. J. Reid and R.G., manuscript in preparation). For the data reduction we first coadded ≤ 0.5 s integration time frames (≤ 0.8 arcsec seeing) with the simple shift-and-add (SSA) algorithm²³, using the pixel position (1 pixel = 0.05 arcsec) of the brightest speckle of IRS7 or IRS16NE.

Next we 'CLEAN'ed the SSA images ($\geq 10^3$ frames) with the Lucy-Richardson²⁴ algorithm using IRS7 as the reference, and reconvolved the result with a 0.15 arcsec FWHM gaussian. We analysed ~ 60 independent data sets from observing runs in 1992.25, 1992.65, 1993.65, 1994.27, 1995.6 and 1996.25. Relative pixel offsets from IRS16NE were calculated from a cross-correlation to the central 9 pixels of each star. Zeroth order (centroid), first-order (rotation angle and pixel scale) and the three second-order instrumental parameters were determined for each coordinate and



data set by comparison to a 'reference frame' constructed from the 1994.27 data. The 2×6 instrumental parameters were determined by solving an over-determined nonlinear equation for N stars via orthonormalization of the $12 \times N$ matrix, resulting in stable solutions for $N \leq 20$. After correction the typical final position fit errors were 0.012 arcsec per data set for the brighter, isolated stars. S1 denotes the high velocity star in the Sgr A* (IR) cluster discussed in the text.

TABLE 1 Derived stellar velocities

Source	K (mag)	$\Delta\alpha$ (")	$\Delta\delta$ (")	ρ_{SgrA} (")	v_{RA} (km s ⁻¹)	v_{Dec} (km s ⁻¹)	v_{rad} (km s ⁻¹)	$ v_{\text{tot}} $ (km s ⁻¹)	Stellar type
	12.5	0.55	0.7	0.89	-450 ± 80	210 ± 80		≥ 500	
	13.5	-0.94	0.23	0.97	-330 ± 80	-420 ± 80		≥ 530	
IRS16NW	10	0.00	1.06	1.06	280 ± 50	70 ± 50	-35 ± 70	290	He I
IRS16C	9.5	1.19	0.25	1.22	-270 ± 50	300 ± 40	150 ± 70	430	He I
	13	-1.45	-0.39	1.50	-60 ± 70	-150 ± 60		≥ 160	
	13	0.40	1.47	1.52	380 ± 80	40 ± 70		≥ 380	
IRS16SW	9.5	0.95	-1.20	1.53	360 ± 70	70 ± 70	400 ± 70	540	He I
	13	-1.75	-0.6	1.85	0 ± 80	-20 ± 50		≥ 20	
	11	0.63	-1.78	1.89	110 ± 50	-260 ± 50	-209 ± 30	350	Late type
	13	-1.05	-1.62	1.93	-90 ± 50	0 ± 50	100 ± 25	130	Late type
IRS16CC	10.5	2.04	0.30	2.06	50 ± 50	250 ± 30		≥ 250	He I?
IRS29S	11.5	-1.88	0.85	2.06	15 ± 50	210 ± 50	-93 ± 20	230	Late type
	11.5	1.96	-0.85	2.14	-10 ± 60	-20 ± 40	-140 ± 30	140	Late type
IRS29N	10	-1.65	1.36	2.14	190 ± 60	-20 ± 30	-110 ± 150	220	He I
	12	-0.91	1.99	2.19	-310 ± 50	0 ± 50	-20 ± 100	310	He I
IRS16SE(1)	11	1.76	-1.40	2.25	290 ± 60	90 ± 50	450 ± 150	540	He I
	13	-1.05	-2.10	2.35	-230 ± 50	-70 ± 70		240	
	10.5	-0.17	-2.37	2.38	50 ± 40	-150 ± 50		≥ 160	
	13.5	2.34	-0.54	2.40	-40 ± 70	240 ± 60		≥ 240	
	10.5	1.15	-2.11	2.40	380 ± 70	-80 ± 60		≥ 390	
	13	-1.85	-1.78	2.57	120 ± 90	-70 ± 50		≥ 140	
	12	-1.14	2.65	2.88	-130 ± 50	-140 ± 30	20 ± 30	190	Late type
	12.5	1.24	2.62	2.90	170 ± 70	60 ± 40	-100 ± 30	210	Late type
	13	-1.86	-2.37	3.01	-90 ± 60	70 ± 40		≥ 110	
IRS16NE	9	2.90	0.90	3.04	250 ± 90	-500 ± 90	10 ± 70	560	He I
IRS16SE(2)	11.5	2.90	-1.49	3.26	125 ± 40	-20 ± 40		≥ 130	He I
IRS33E	10	0.50	-3.38	3.42	210 ± 60	-60 ± 30	60 ± 70	230	He I
	13.5	-1.80	-2.95	3.46	80 ± 80	110 ± 60		140	
IRS33W	11	-0.50	-3.43	3.47	60 ± 60	30 ± 35	80 ± 25	104	Late type
IRS21	10	2.36	-3.05	3.86	-140 ± 50	50 ± 40		≥ 150	Embedded
IRS34W	10.5	-4.08	1.62	4.39	-80 ± 50	-130 ± 40	-230 ± 70	280	He I
IRS3	11.5	-2.31	3.79	4.44	170 ± 40	110 ± 40		≥ 200	Embedded
	13.5	-3.70	2.88	4.69	-60 ± 100	-200 ± 60		≥ 210	
IRS7	6.5	0.20	5.55	5.55	0 ± 60	-150 ± 60	-130 ± 20	200	Late type
IRS7W	13	-3.91	5.00	6.35	70 ± 60	-100 ± 60	-360 ± 80	380	He I
	12.5	-6.30	4.60	7.80	20 ± 60	-40 ± 60		≥ 45	
	12.5	-7.10	3.75	8.03	-70 ± 70	-120 ± 60		≥ 140	
BHA4E	11.5	-5.70	5.67	8.04	60 ± 60	70 ± 30	-80 ± 30	120	Late type
BHA4W	11.5	-6.45	6.02	8.82	-270 ± 80	170 ± 40	100 ± 30	330	Late type

time, along with the fitted proper motions. Their locations can be seen from the accompanying grey-scale 2.2- μm image which also shows the best-fit proper-motion vectors of other stars of Table 1. Of the 39 stars listed in Table 1, proper motions at the $\geq 5\sigma$ level are detected in at least one coordinate in 10 cases and at the $\geq 4\sigma$ level in 19. The selection criteria for including stars in Table 1 were that they are not located in a confused region, and that they produced repeatable positions in all six epochs with proper-motion velocity errors not exceeding 100 km s^{-1} . This simultaneously constrained the selection to stars brighter than K-band magnitudes of ~ 13.5 .

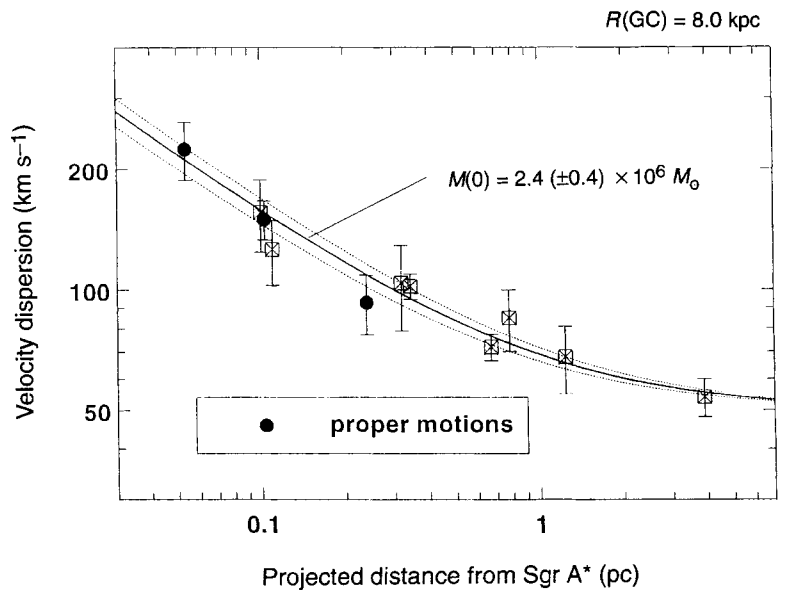
A simple consideration shows that the motions we have detected cannot be due to orbital motion in multiple star systems that are not resolved at 0.15-arcsec resolution. Taking the average space velocity of the He I stars ($\sim 400 \text{ km s}^{-1}$) in Table 1 as an orbital velocity, and assuming further that a typical star has a mass of $60 M_{\odot}$ (ref. 5) the orbital period of a binary would be about a week with an orbital radius of $\sim 50 R_{\odot}$, comparable to the actual photospheric diameters of the He I stars⁵. If this were truly the case there should be large variations of the relative positions within the typically 10 days of a given epoch, but no positional changes on a scale of several years. Turning the argument around, orbital periods of three or more years at the observed velocities would require unrealistic stellar masses approaching $10^4 M_{\odot}$. Variability in two or more stars that are very close in projection may also lead to apparent proper motion. A number of stars indeed appear to show some flux variations in our data sets and we thus cannot exclude that variability may in fact corrupt the derived motions of

a few of the stars in the sample. But as we have mostly considered only relatively bright isolated stars with six-epoch coverage and have discarded all stars with $\geq 100 \text{ km s}^{-1}$ errors, we can exclude the possibility that variability can cause the motions we show here. In addition, lunar occultation measurements^{14,15} have proved that the brightest members of the IRS16 complex (with the best proper motion data) are very probably single stars.

We thus conclude that we have detected orbital proper motions of the stars in the central gravitational field. An immediate important conclusion is that the velocity dispersion σ is very similar in all three coordinates and hence any anisotropy of the stellar motions must be small. This can be best seen from Fig. 2, where we have plotted radial velocity (from refs 6, 7, 16–18) and proper-motion velocity dispersions as a function of projected radius p from Sgr A*. Table 1 also shows that this conclusion holds if only stars are considered for which all three velocities are available. Taken together, the measurements for $p \geq 0.053 \text{ pc}$ now show a very significant keplerian ($\sigma(p) \approx p^{-0.5}$), falloff of the stellar velocities with projected distance p from the dynamic centre (assumed to be at Sgr A*), demonstrating convincingly that there must be a central, compact and dark⁷ mass concentration.

Next we turn to the constraints on the mass distribution the new data provide. Fitting to the projected velocity dispersion data in Fig. 2 a model with a central point mass plus an extended isothermal cluster of dispersion at large radii of $\sim 50 \text{ km s}^{-1}$ (providing an excellent fit to stellar mass data at $p \geq$ a few parsecs, see ref. 7 for details), we find that a central mass of $2.4 \times 10^6 M_{\odot}$ is

FIG. 2 Projected stellar velocity dispersions as a function of projected distance from Sgr A*. Filled circles (and 1σ statistical error bars) are the dispersions (corrected for measurement bias⁷ derived from the present proper motion data for 39 stars between 0.004 and 0.34 pc (RA and dec. motions combined and separated in four independent groups of between 9 and 20 stars each). Open rectangles are radial velocity dispersion data^{7,16–18}. For the radial velocity dispersion of the early-type stars, a 120 km s^{-1} counter-rotation was first subtracted from the velocities and then statistically re-introduced as an effective radial velocity dispersion ($\sigma_{\text{eff}}^2 = \sigma_r^2 + v_{\text{rot}}^2/3$; ref. 7) where σ_r is the radial velocity dispersion and v_{rot} is the rotation velocity. The fact that the innermost radial velocity dispersion of the late-type stars at 0.11 pc ($\sigma_r = 126 \pm 23 \text{ km s}^{-1}$) is lower than either the radial velocity dispersion of the early type stars or the proper-motion dispersion at that radius is probably due to the central 'hole' in the distribution of late-type stars^{4,6,7}. Most of the late-type stars seen projected toward the central few arcseconds are actually at much larger radii so that the projected velocity dispersion appears low; after correction for this effect the dispersion would be $\sim 150 \text{ km s}^{-1}$, in agreement with the other two data points. The solid curve is the best fit of the stellar dynamics with an isotropic velocity field in the potential of a point mass ($M(0)$) plus an isothermal cluster with one-dimensional dispersion at infinity of 50 km s^{-1} . This dispersion (which is added in squares to the dispersion caused by the central dark mass) accounts for the background mass of the (visible) stellar cluster⁷. The



dotted curves represent the $\pm 1\sigma$ uncertainties ($\pm 0.4 \times 10^6 M_{\odot}$). The assumed distance to the Galactic Centre was $R(\text{GC}) = 8.0 \text{ kpc}$ (ref. 25).

required with high significance. The 1σ statistical uncertainty of this estimate is $\pm 0.35 \times 10^6 M_{\odot}$, with an additional systematic error resulting in a total uncertainty of $\pm 0.5 \times 10^6 M_{\odot}$. Proceeding as in ref. 7 we have also calculated enclosed masses from the Bahcall–Tremaine and virial theorem projected mass estimators. The proper-motion data indicate a mass of $(2.5 \pm 0.5) \times 10^6 M_{\odot}$ within a radius of 0.16 pc (40 velocities), and $(2.66 \pm 0.8) \times 10^6 M_{\odot}$ within 0.083 pc of the dynamic centre (20 velocities). Jeans equation mass modelling (as in ref. 7) of the combined radial and proper motion data at $P \geq 0.053 \text{ pc}$ implies that the central dark mass has a density of at least $6.5 \times 10^9 M_{\odot} \text{ pc}^{-3}$ and a core radius $< 0.035 \text{ pc}$. An analysis of the individual proper-motion vectors in Fig. 1 with the assumption that they represent simple circular orbits indicates that the dynamic centre of the orbiting stars is within 0.015 pc of Sgr A*.

We now consider the central group of stars in the immediate vicinity of the compact radio source Sgr A* (indicated by a cross in Fig. 1). The sources in the very important central Sgr A*(IR) cluster^{9–11} are crowded and relatively faint. Considering only those stars clearly detected in all three epochs indicates that at least one star (labelled S1 in Fig. 1) in the Sgr A*(IR) cluster moves consistently from epoch to epoch. From our data we derived a preliminary velocity of $> 1,600 \text{ km s}^{-1}$ towards the southeast. Although the individual motions within the central Sgr A*(IR) cluster are still relatively poorly determined and therefore not included in Table 1 the indicated velocity dispersion of the group is large. A detailed analysis of the central cluster is given elsewhere¹³. The significance of this possible result could be confirmed by expanding the time baseline of our measurements, or by repeated observations with other large-aperture telescopes. If confirmed, this result then would suggest that the central mass is concentrated and centred within $\leq 0.009 \text{ pc}$ of Sgr A* and that the mass density is possibly more than two orders of magnitude higher than what we derived above.

The Galactic Centre, along with the 'mega-maser' galaxy NGC4258^{19,20}, are now known to contain dark central masses with densities significantly exceeding $10^9 M_{\odot} \text{ pc}^{-3}$. What is the nature of this dark mass? Given our detailed knowledge about

the stellar content of the Galactic Centre, we can now exclude with some confidence that the mass concentration is a cluster of solar-mass remnants (neutron stars or white dwarfs⁷). Considering the stellar dynamics outside $\sim 0.05 \text{ pc}$, the remaining possibilities are a core collapsed cluster of $10\text{--}20 M_{\odot}$ stellar black holes^{21,22}, or a single massive black hole, or a combination thereof. Including the Sgr A*(IR) cluster evidence, a single massive black hole is the most likely configuration. □

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CORRESPONDENCE should be addressed to R.G. (e-mail: genzel@mpe-garching.mpg.de).

Spectroscopic evidence for interstellar ices in comet Hyakutake

William M. Irvine, Dominique Bockelee-Morvan*, Dariusz C. Lis†, Henry E. Matthews‡§, Nicolas Biver*, Jacques Crovisier*, John K. Davies‡, William R. F. Dent‡, Daniel Gautier*, Peter D. Godfrey||, Jocelyn Keene†, Amy J. Lovell, Tobias C. Owen||, Thomas G. Phillips†, Heike Rauer*, F. Peter Schloerb, Matthew Senay|| & Kenneth Young†

Five College Radio Astronomy Observatory, 619 LGRC, University of Massachusetts, Amherst, Massachusetts 01003, USA

* Observatoire de Paris-Meudon, F-92195 Meudon Principal Cedex, France

|| Chemistry Department, Monash University, Clayton 3168, Australia

† Institute for Astronomy, University of Hawaii, 2680 Woodlawn Drive, Honolulu, Hawaii 96822, USA

‡ California Institute of Technology, Downs Laboratory of Physics 320-47, Pasadena, California 91125, USA

§ Herzberg Institute of Astrophysics, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada

‡ Joint Astronomy Centre, 660 N. A'ohoku Place, University Park, Hilo, Hawaii 96720, USA

VOLATILE compounds in comets are the most pristine materials surviving from the time of formation of the Solar System, and thus potentially provide information about conditions that prevailed in the primitive solar nebula¹⁻³. Moreover, comets may have supplied a substantial fraction of the volatiles on the terrestrial planets, perhaps including organic compounds that played a role in the origin of life on Earth⁴⁻⁶. Here we report the detection of hydrogen isocyanide (HNC) in comet Hyakutake. The abundance of HNC relative to hydrogen cyanide (HCN) is very similar to that observed in quiescent interstellar molecular clouds, and quite different from the equilibrium ratio expected in the outermost solar nebula, where comets are thought to form. Such a departure from equilibrium has long been considered a hallmark of gas-phase chemical processing in the interstellar medium⁷, suggesting that interstellar gases have been incorporated into the comet's nucleus, perhaps as ices frozen onto interstellar grains. If this interpretation is correct, our results should provide constraints on the temperature of the solar nebula, and the subsequent chemical processes that occurred in the region where comets formed.

The identification of HNC was made in comet C/1996 B2 (Hyakutake) through its $J = 4-3$ transition at 362.630092 GHz. The first detection was obtained at the James Clerk Maxwell Telescope (JCMT) on March 16.6 UT when the comet was at 1.219 AU from the Sun and 0.305 AU from the Earth. Observations quickly organized at the Caltech Submillimeter Observatory (CSO) on March 22.5, when the comet was closer to the Earth (0.137 AU), allowed confirmation of the line with a high signal-to-noise ratio. Both sets of hydrogen isocyanide (HNC) observations were preceded by measurement of the hydrogen cyanide (HCN) $J = 4-3$ line at 354.505472 GHz, from which an abundance with respect to the principal volatile in the nucleus (water) of $\text{HCN}/\text{H}_2\text{O} \approx 0.0016$ may be estimated⁸. The pointing was monitored regularly using continuum sources (measured pointing shifts $\leq 3.5''$), and the cometary ephemeris was checked by performing small maps of the HCN distribution. The spectra are shown in Fig. 1; observed line intensities are given in Table 1.

Because the rotational constants and electric dipole moments of the closed-shell species HNC and HCN are very similar, the energy level populations for these two isomers are thought to be nearly equal when excitation is dominated by collisions (the situation in dense interstellar clouds^{9,10}). This case should apply

close to the nucleus in the cometary coma, so that the ratio of intensities observed for a given transition will closely approximate the ratio of abundances, apart from possible effects of optical depth for the more abundant species, HCN (these effects are not large; see Table 1 legend). The HNC/HCN abundance ratio is thus largely independent of assumptions that are necessary to calculate absolute molecular production rates, unless the distribution of these species in the inner coma is quite different (we return to this possibility in point 6 below). Farther from the nucleus the excitation will be dominated by the solar radiation field and fluorescence equilibrium may be approached¹¹; however, this does not seem to be a major effect for the beam sizes employed here (Table 1 legend).

The ratio of the integrated areas (I) under the $J = 4-3$ line profiles for HCN and HNC from the JCMT observations was $I(\text{HNC})/I(\text{HCN}) = 0.074 \pm 0.015$, whereas the CSO observations on 22 March obtained $I(\text{HNC})/I(\text{HCN}) = 0.092 \pm 0.014$. Correcting for optical depth (line saturation) of the HCN transition produces an abundance ratio $\text{HNC}/\text{HCN} \approx 0.06$ on both dates. This value is approximately equal to the abundance ratio HNC/HCN found in interstellar clouds which have a kinetic temperature of the order of 50 K and is consistent with the upper limit of 0.3 that was found for comet Halley^{12,13}. In contrast, the chemical equilibrium ratio at 200 K, the expected near-nucleus coma temperature, is less than 10^{-15} , and is even less at lower temperatures¹⁴. As far as we can determine, the presence of a significant amount of HNC in a comet had not been predicted, presumably because the higher densities in the solar nebula relative to interstellar clouds would tend to drive the chemistry closer to equilibrium abundances. How may we account for the present observations? We consider several possibilities.

(1) The HNC may be surviving interstellar molecular material originally produced by gas-phase chemistry. In interstellar clouds the gas-phase abundance ratio HNC/HCN can exceed unity in cold (10 K) clouds; it also appears to exhibit a marked dependence

TABLE 1 Parameters of the observed spectra

Date (UT)	r (AU)	Δ (AU)	Transition	Line area (K km s^{-1})
JCMT	1.22	0.31		
March 16.50			HCN $J = 4-3$	5.41 ± 0.07
March 16.63			HNC $J = 4-3$	0.40 ± 0.06
CSO	1.10	0.14		
March 22.47			HCN $J = 4-3$	16.47 ± 0.08
March 22.51			HNC $J = 4-3$	1.51 ± 0.09

Observations towards presumed position of nucleus; intensity scale is main-beam brightness temperature using efficiencies of 0.58 (JCMT) and 0.7 (CSO); r is heliocentric distance; Δ is geocentric distance; date is average during observation; integrated intensities computed over a 2 km s^{-1} velocity interval for the JCMT lines, and over 3 km s^{-1} and 5 km s^{-1} intervals for the CSO lines of HNC and HCN, respectively (the larger value employed to include all observed HCN hyperfine components). Quoted uncertainties do not include calibration uncertainties, estimated at 10%. For CSO observations (Lis *et al.*, manuscript in preparation), an optical depth of 0.7 at line centre has been estimated by comparing the strengths of the stronger to the weak hyperfine components of the HCN $J = 4-3$ transition. This lowers the abundance ratio from the optically thin value of 0.09 to $\text{HNC}/\text{HCN} \approx 0.06$. Opacity effects were probably less important at the time of the JCMT observations, owing to the larger area in the coma observed and the lower gas production and hence much lower column density; we estimate that the HNC/HCN abundance is indistinguishable from the later (CSO) value of 0.06. Another potentially complicating factor is the effect of radiative excitation and fluorescent decay in the solar radiation field, which will dominate collisional excitation in the outer coma. However, telescope beam sizes corresponded to only 2,000 km (CSO) and 3,000 km (JCMT) in the coma. Calculations with the HCN excitation model of Crovisier³⁵ show that most of the observed emission should have arisen from the collision-dominated region. The relative abundances of HNC and HCN should then not be overly complicated by effects of radiative excitation.

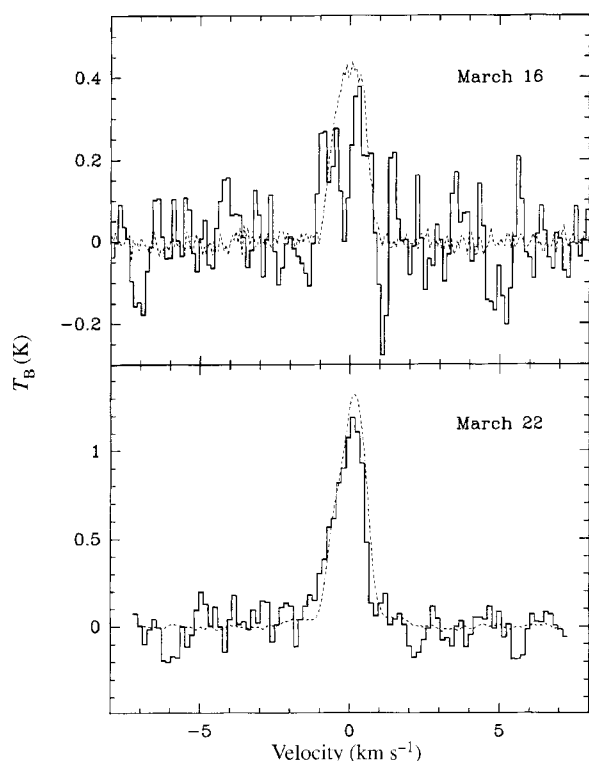


FIG. 1 The $J(4-3)$ line of hydrogen isocyanide (HNC; solid lines) and the corresponding $J(4-3)$ line of HCN (dashed lines, spectra multiplied by 0.1) observed on 16 March 1996 at the James Clerk Maxwell Telescope (top), and on 22 March 1996, at the Caltech Submillimeter Observatory (bottom; Table 1 legend). The velocity scale is with respect to the nucleus. Spectral resolutions are 0.13 km s^{-1} and 0.16 km s^{-1} for the top and bottom spectra, respectively. The intensity scale is main-beam brightness temperature (T_B).

on local kinetic temperature (T_k), with values decreasing to around 0.005 for $T_k \approx 200 \text{ K}$ (refs 10, 12, 15). A relatively high abundance ratio is predicted by the kinetics of gas-phase, ion-molecule chemistry, although a number of uncertainties remain concerning branching ratios of relevant reactions, the cause of the putative temperature dependence, and so on^{7,16-18}. Moreover, models suggest that the large gas-phase ratios can be retained as the molecules freeze out onto dust grains, although some decrease in the ratio might occur through processes in the grain mantles¹⁹. According to the model of Greenberg²⁰, cometary nuclei are agglomerates of largely unprocessed interstellar grains. The present HNC/HCN ratio is consistent with this model, if gas-phase HNC and HCN condensed onto such grains in the interior of the cloud that ultimately contracted to form the solar nebula and then survived without isomerization, first in the grain mantles, then during the formation of the solar nebula, and subsequently for the 4.5 Gyr lifetime of the Solar System, and finally during the processes associated with production of the cometary coma. Although neither HCN nor HNC has unequivocally been identified in the icy mantles of grains in dense interstellar clouds, such grains do exhibit an absorption feature at $4.62 \mu\text{m}$ which may arise from either nitriles or isonitriles ($^1\text{XCN}^*$)^{21,22}.

(2) HNC might be produced by irradiation of an icy matrix containing HCN. This could conceivably occur either in the mantles of interstellar grains, in grains within the solar nebula, or near the surface of the cometary nucleus after its formation. Ultraviolet irradiation of HCN in an argon matrix is known to produce HNC, and in fact was the technique used in early laboratory characterization of the HNC molecule²³. If this mechanism were operative, it would imply the presence in cometary nuclei of the much more complex organics produced in

laboratory irradiation of interstellar ice analogues^{3,24}. However, ultraviolet and particle radiation should only penetrate the outer few micrometres to millimetres of the nuclear ices². Thus, if irradiation of ices is the source of HNC, it would be much more effective before incorporation of interstellar grains into a cometary nucleus, because the entire grain mantle could then be subject to such processing. Thus, in this case the HNC would still probably be of interstellar origin.

(3) HNC might be produced by non-equilibrium chemical processes in the solar nebula itself, whatever the prior history of the nebular material. Various sources of energy are available, including ultraviolet photons, lightning, shock heating and solar flares²⁵. Processes similar to those active in interstellar clouds might play a role. Existing models have not, to our knowledge, considered the production of HNC. In fact, some consider that HCN itself is probably surviving, unprocessed interstellar material, rather than a nebular product². If the cometary HNC could be shown to have been produced in the outer solar nebula, it might imply that other extreme departures from thermochemical equilibrium (such as the large hydrogen isotope fractionation observed in meteoritic organic matter²⁶) might be the result of nebular rather than interstellar processes.

Finally, we must consider whether HNC might be produced in the cometary coma directly. There are various possible mechanisms.

(4) Could HNC be produced by gas-phase processes in a manner similar to that which occurs in the interstellar medium? At the nucleus of a typical comet at 1 AU from the Sun, the gas temperature and density are, respectively, about 200 K and 10^{13} cm^{-3} , several times and many orders of magnitude greater than the corresponding values in quiescent molecular clouds²⁷. However, the rapid expansion in the coma leads, according to models, to a drop in temperature within several hundred kilometres of the nucleus to about 50 K, while the density decreases as the inverse square of the distance from the nucleus²⁷. When the solar radiation field and the radiative and chemical effects of cometary grains are taken into account, the resulting chemistry becomes extremely complicated²⁸. It may be that ions such as H_2O^+ and H_3O^+ produced by solar ultraviolet irradiation would charge transfer to HCN, leading to HCNH^+ , which (as in the interstellar models) could undergo dissociative electron recombination to produce HNC, HCN and CN.

(5) The solar radiation field, which is of course absent in the interstellar case, might produce novel effects in the cometary environment. It has been calculated²⁹ that infrared relaxation of HCN from vibrational levels of the ground electronic state with more than $\sim 2 \text{ eV}$ of internal energy leads to an HNC/HCN ratio of ~ 0.05 , essentially equal to our result. Radiative decay from the bent excited electronic states³⁰ of HCN would tend to populate these vibrational levels. Thus, if the cross-section for ultraviolet absorption by HCN needed to populate the excited electronic states were sufficiently large, the observed HNC might be the product of such photochemistry. Present indications, however, are that the rate of non-dissociative excitation of the electronic bands of HCN at 1 AU in the solar radiation field is much less than both the photodissociation rate and the infrared vibrational excitation rate, and hence probably not significant³¹.

(6) A more familiar process in the coma for producing an unstable species such as HNC would be photodissociation of a heavier molecule; the radicals that dominate the optical spectra of comets are such 'daughter species'. The similar line shapes for HCN and HNC, resulting from Doppler broadening due to mass motion in the coma, argue against this possibility. We also note that, if HNC were a daughter product, its extended spatial distribution in the coma would lead to a calculated abundance much higher than that estimated here.

We consider that careful theoretical analysis, new laboratory experiments, and further astronomical observations will be necessary to sort out the implications of the present results. For example, careful modelling of coma chemistry for the deduced

parameters appropriate to comet Hyakutake should be carried out, in order to estimate an HNC/HCN ratio for varying compositions of the nuclear ices. Several questions may need laboratory investigation: would HNC trapped in the nuclear H₂O matrix at ≤ 20 K survive isomerization to HCN during the 4.5-billion-year history of the Solar System? How much HNC is produced by irradiation of HCN in a water-ice matrix? Would HNC survive in H₂O ice heated to ~ 200 K as the comet approaches the Sun and the ices sublimate? Are the cross-sections for excitation of HCN to the excited electronic states indeed too low to be significant in the solar radiation field at 1 AU heliocentric distance? What are the dissociation cross-sections and branching ratios for possible parent molecules of putative daughter HNC in the coma? On the observational side the current approach of comet Hale-Bopp provides opportunities for seeking variations of the HNC/HCN ratio with both heliocentric distance and position in the coma, which would be expected if HNC is produced by photochemistry. Measurement of the DCN/HCN and DNC/DCN abundance ratios would allow comparison with interstellar values: both HCN and HNC can show deuterium enhancements of up to a factor of 1,000 with respect to the cosmic D/H ratio³¹, and a large deuterium fractionation would significantly strengthen the case for the survival of interstellar HCN and/or HNC in comets. Note that the HDO/H₂O ratio in comet Halley is about twice the terrestrial value and an order of magnitude larger than the protosolar value, providing strong evidence for the survival of water from interstellar grain mantles³² (the comet Halley water *ortho/para* ratio also appears to support an interstellar origin²). HDO has been detected in comet Hyakutake^{33,34}, but a firm determination of the D/H fractionation is premature at this time. □

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CORRESPONDENCE should be addressed to W.M.I. (e-mail: irvine@fcrs1.phast.umass.edu).

Possible contribution of a metal-rich magmatic fluid to a sea-floor hydrothermal system

Kaihui Yang & Steven D. Scott

Scotiabank Marine Geology Research Laboratory, Department of Geology, University of Toronto, Toronto, Canada M5S 3B1

THE source of the hydrothermal fluids vented in active volcanic areas on the sea floor^{1–3} has been a matter of some debate^{4–7}; they may arise purely from the interaction of circulating sea water with the hot rocks through which it passes^{1,3,8}, or there may be an admixture of a fluid escaping from magma at depth, as is seen in subaerial geothermal systems⁹. The answer to this question also bears on the origin of the sulphide ores deposited by sea-floor hydrothermal systems, and their ancient analogues^{8,10,11} preserved on land. Here we present direct evidence for the presence of magmatic fluid in the lavas that host an actively forming massive sulphide deposit in the eastern Manus back-arc basin. We find high concentrations of chlorides and sulphides of ore-forming metals such as copper, zinc and iron in CO₂-rich gaseous bubbles found both in melt inclusions trapped in the phenocrysts of the volcanic rocks, and in the matrix glass. We conclude that a metal-rich fluid was present in the magma before eruption, and probably exsolved as the pressure decreased. This finding suggests the possibility for the contribution of large quantities of ore-forming metals to a sea-floor hydrothermal system.

A hydrothermal leaching model, in which ore metals are derived from reactions between sea water and rock at high temperature, has been proposed for the formation of ancient volcanogenic massive sulphide ores⁸. But the close spatial association between massive sulphides and the volcanic rocks of both ancient and modern sea floors^{4–8,11} suggests that a possible magmatic contribution to the ore-forming fluid should also be taken into account. Magmatic fluids, capable of carrying abundant ore metals and volatiles^{4,7,12} and escaping from magma on depressurization^{4,5}, are well known from modern active volcanoes on land and have been postulated for various types of ancient ores^{4,9}.

This potential contribution of volatiles and ore metals from a magma to a sea-floor hydrothermal system can be demonstrated if (1) a fluid phase can be formed by the exsolution of volatiles from an evolving magmatic system; (2) ore metals are enriched in that fluid phase; and (3) that fluid can be separated and mixed with the seawater convective system. The first two of these can be approached by the study of melt inclusions in the phenocrysts and vesicular matrix glass of fresh volcanic rocks that host a hydrothermal system on the sea floor. Melt inclusions are samples of magma that were trapped in crystals at the high temperature and pressure of the magma chamber and, therefore, record the behaviours of metals and volatiles in pre-erupted magma¹². The vesicular volcanic glass was quenched from a high-temperature magma and thus records the degassing processes¹³.

We have assessed the potential for a magmatic contribution to an active, modern, submarine hydrothermal system by a study of dredged volcanic rocks that host the Cu–Zn–Pb–Ag–Au polymetallic sulphide deposits in eastern Manus basin offshore eastern Papua New Guinea in the western Pacific¹⁴. These deposits occur in the PACMANUS hydrothermal field, a modern analogue of the

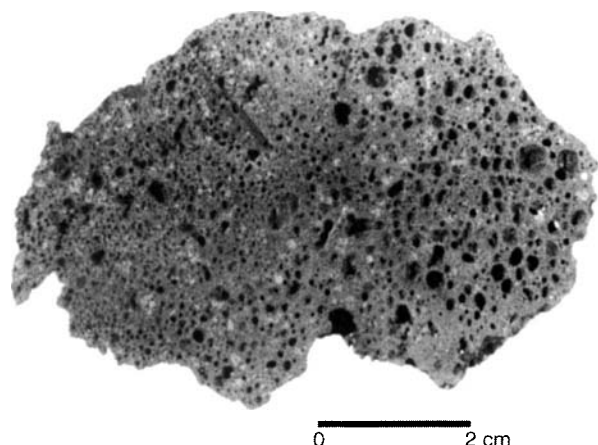


FIG. 1 Vesicular andesite sample dredged from the volcanic ridge (1,675–2,000 m water depth) hosting the actively forming PACMANUS polymetallic sulphide deposits in eastern Manus basin, western Pacific Ocean. The samples for this study are very fresh, and may be the volcanic rocks of most recent eruption. The rock consists of phenocrysts (light grey spots) or pyroxene and lesser olivine, and vesicular matrix glass. Electron microprobe analyses show that the composition of the matrix glass varies from andesitic basalt to andesite (52–62% SiO_2). The vesicles were formed by the exsolution of volatiles from a magma that ascended onto the sea floor. The vesicularity is 33.2 ± 3.9 vol.%, measured by digital image analysis (IMAGE Pro-Plus).

hydrothermal processes that formed massive sulphide deposits in the ancient geological record, which is presently producing massive sulphides on a predominantly felsic volcanic ridge near 1,700 m water depth^{15,16}. Bulk analyses of the volcanic rocks define a calcic fractionation trend ranging from andesite to rhyolite, which was probably derived from a single, relatively shallow, differentiating magma¹⁵. The rocks are vesicular (Fig. 1), massive or glassy, and microporphyrific, containing phenocrysts of pyroxene, plagioclase, olivine, minor magnetite and ilmenite, and supported by microlites and glass.

The melt inclusions in phenocrysts (Fig. 2) are glassy, round or irregular blebs, with sizes ranging from <1 to 500 μm , but mostly 30–60 μm . Typically, a melt inclusion contains silicate glass, minerals and one to eight bubbles varying in size from 5 to

20 μm across. The bubbles occupy 10–40 vol.% of the inclusion, similar to that of the matrix glass (33.2 ± 3.9 vol.%, Fig. 1). The extensive occurrence of bubbles in both the matrix glass and the melt inclusions demonstrates that a volatile phase exsolved from the magma¹³. A peak observed in infrared spectra at $2,350\text{ cm}^{-1}$ indicates that the major constituent of the bubbles is CO_2 , although its concentration could not be quantified owing to the poor constraint on the infrared path length in the bubbles. The gas contains H_2O , but the lack of a liquid phase in the bubble and a much smaller H_2O peak in the infrared spectra indicate that it is subordinate to CO_2 . This CO_2 -dominated volatile phase, as also documented in other volcanic and plutonic rocks on the modern sea floor^{17,18}, active volcanoes¹⁹ and experiments^{20,21}, was probably derived from the early stage of an open-system degassing^{18–21}.

Irregular, amorphous precipitates (Fig. 2) are observed typically to comprise 1–10 vol.% (Table 1), averaging 4.7 vol.% or 26.5 wt%, of the CO_2 -filled bubbles in both melt inclusions and the matrix glass. Electron microprobe analyses indicate that the precipitates (in 70 bubbles examined) contain mainly Cu, Zn, Ni, Fe, Na, S and Cl (Fig. 3), probably as sulphides and chlorides. Although quantitative analysis is not possible owing to the difficulty of calibration, micro-analyses (24 points) of the precipitates in various bubbles gave a relatively consistent ratio (<20% variability) among the major elements: $\text{Zn}:\text{Cu}:\text{Fe}:\text{Ni}:\text{S}:\text{Cl}:\text{Na} = 1:3.2:1.2:0.5:1.2:3.5:1.1$. Using these data, the CO_2 -rich gaseous phase is estimated to contain an average (<35% uncertainty) of 2.3 wt% Zn, 7.2 wt% Cu, 2.7 wt% Fe, 1.1 wt% Ni, 2.7 wt% S, 7.9 wt% Cl and 2.5 wt% Na. Fresh, degassed dacite, differentiated from a basalt-andesite magma at PACMANUS, contains only 79 p.p.m. Zn, 57 p.p.m. Cu and 5 p.p.m. Ni (ref. 15), so these metals were extremely partitioned into a CO_2 -, Cl- and S-bearing gaseous phase of the magma. Elsewhere, a copper-rich volatile phase has been found in melt inclusions in quartz from rhyolitic lava^{22,23} and in fluid inclusions in quartz from cassiterite veins in a granite intrusion²⁴. Besides Cu, this study also demonstrates the volatility of Zn, Ni, Fe and Na. The discovery of this metal-rich volatile phase confirms the feasibility of metal transport by high-temperature fluids in a magma, as anticipated by experimental studies^{25,26} and observations of subaerial volcanoes^{27,28}.

Because the metal- and CO_2 -rich phase of the melt inclusions was trapped in phenocrysts that crystallized early in the andesite-basaltic magma, there is direct evidence for the presence of a high-temperature, ore-metal-rich fluid in the pre-erupted magma at

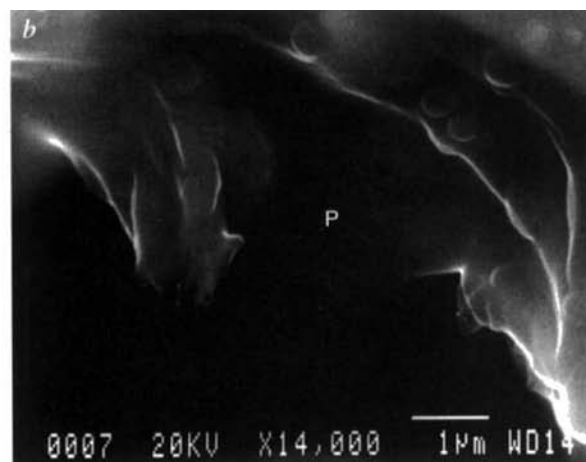
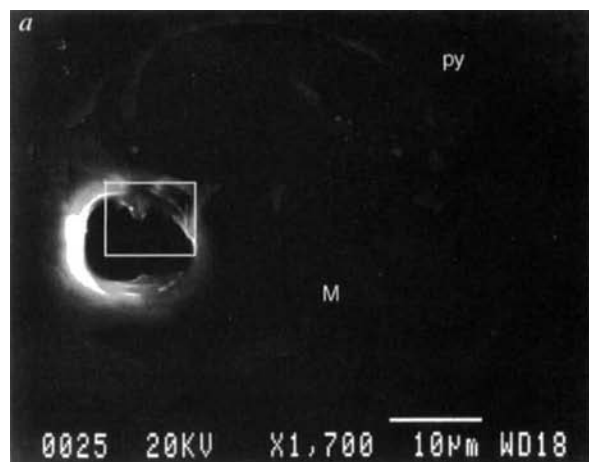


FIG. 2 Scanning electron micrographs: a, melt inclusion (M) with two bubbles, one to the left and the other at the upper right, in pyroxene (py) of andesite. The composition of the silicate glass in the melt inclusions varies from andesitic basalt to andesite (52–60% SiO_2), which is similar to the composition of the matrix glass (Fig. 1) of the samples. The larger bubble contains ore-metal-rich precipitates on its wall. b, Magnified portion of the large bubble in a showing the precipitates (P). The precipitates, irregularly accumulated on the wall of the bubble, exhibit on their surface small, round

'droplets' with sizes about 0.1–0.5 μm across. We interpret these 'droplets' to be condensates from the high-temperature magmatic fluid as the temperature decreased. This 'droplet' texture is typical of the precipitates in the CO_2 -rich bubbles both in the melt inclusions and in the matrix glass of the volcanic rocks. These irregular, amorphous precipitates are distinctly different from the evenly spaced metal sulphide spherules within vesicles in basalt described by Moo and Calk³⁷.

PACMANUS. The contribution of magmatic volatiles (such as CO_2) to hydrothermal systems has been demonstrated by the direct analyses of hydrothermal fluids venting onto the sea floor of the Pacific Ocean^{29,30}. The vesicular texture (Fig. 1) of the volcanic rocks at PACMANUS indicates that magmatic volatiles were significantly separated from the magma through the degassing process. The volatile fraction (X) exsolved from the depressurized magma can be estimated by an equation (modified from Jaupart and Tait³¹): $X = (\rho_g/\rho_l)[\alpha/(1-\alpha)]$, where ρ_g is the gas density at the eruption pressure and temperature (0.07 g cm^{-3} of CO_2 fluid at $1,000^\circ\text{C}$ and 170 bar (ref. 32), $1,700 \text{ m}$ below sea level at PACMANUS); ρ_l is the melt density (2.7 g cm^{-3} ; ref. 33); and α is the gas volume fraction of the erupted magma, which is approximated from the vesicularity of the volcanic rocks by assuming all the volatiles were trapped in the vesicles. This equation gives a minimum estimate of $1.3 \text{ wt}\%$ for X , representing the fraction of volatiles degassed from the magma and available to the hydrothermal system that is producing the 'black smokers' at PACMANUS.

Compared with the dilute hydrothermal fluids (80 p.p.m. Fe, 6 p.p.m. Zn, 2 p.p.m. Cu; average data for 21°N East Pacific Rise)² venting on the sea floor, the magmatic volatile phase trapped in volcanic rocks has a considerably higher concentration of metals, including most of the major ore metals of the PACMANUS massive sulphide deposit (10.9% Cu, 26.9% Zn, 1.7% Pb, 14.9% Fe, 29.5% S, 230 p.p.m. Ag and 15 p.p.m. Au)¹⁶. Such an ore-metal-rich magmatic fluid could potentially contribute significant amounts of Cu and Zn to the hydrothermal systems that form massive sulphide deposits. However, this fluid is not enriched in Pb which is a major metal in the PACMANUS and many other massive sulphide deposits. We speculate that Pb is enriched in fluid associated with more evolved felsic magmas³⁴, or that Pb could possibly be derived entirely from the seawater-rock reaction^{1,8}. Moreover, the studied magmatic fluid transports much Ni which is neither enriched in the PACMANUS or other analogous modern or ancient massive sulphide deposits, nor found in vent

TABLE 1 Volume measurements of CO_2 bubbles and precipitates

Sample	Size ($\mu\text{m} \times \mu\text{m}$)	Volume of bubble V_b (μm^3)	Volume of precipitate V_p (μm^3)	V_p/V_b (vol.%)	W_p/W_b (wt%)
G-2	12×14	1,616	124	7.7	37.6
G-3	58×60	172,586	13,480	7.8	37.9
G-4	31×40	34,552	341	1.0	6.8
G-5	12×17	2,148	119	5.6	30.0
G-6	4×6	102	5.7	5.5	29.6
G-7	17×27	7,372	156	2.1	13.4
G-8	15×17	3,562	369	10.3	45.3
G-9	14×28	5,286	176	3.4	20.3
G-10	14×15	3,118	148	4.7	26.5
G-11	38×48	66,496	1,237	1.9	12.2
G-12	7×9	418	5.5	1.3	8.7
G-14	32×50	28,920	1,667	5.7	30.4
I-1	12×18	2,616	110	3.9	22.7
I-2	12×13	1,668	71	4.3	24.5
I-3	8×9	482	26	5.3	28.8

Measurements are of metal- and CO_2 -rich bubbles in the matrix glass (G-2 to G-14) and in the melt inclusions in pyroxene (I-1 to I-3) of fresh andesite from the PACMANUS hydrothermal field. The matrix glass has been degassed, with bubbles ranging in sizes from <5 to $>10^4 \mu\text{m}$ across (Fig. 1). The large bubbles formed from the coalescence and extreme expansion of small bubbles, and do not represent the initial compressed gaseous phase. The small bubbles are more likely to represent the initial unexpanded magmatic volatile phase, although the volume may have increased slightly due to the shrinkage of the silicate glass as the temperature decreased. We take the measurement of the small bubbles ($<60 \mu\text{m}$ across) in the matrix glass and the bubbles in the melt inclusion as an approximation of the initial magmatic volatiles phase. The volumes of the bubbles and the precipitates were calculated ($<20\%$ uncertainty) on the basis of the measurement of their sizes using an electron microscope with the application of a digital image analysis program (IMAGE PLUS, Media Cybernetics, Silver Spring, MD, USA). The wt% of the precipitates was estimated by assuming that the initial magmatic volatile phase is a supercritical CO_2 fluid (0.4305 g cm^{-3} density at $1,000^\circ\text{C}$ and $1,400 \text{ bar}$)³² and the condensed precipitates are dominantly chlorides (3.15 g cm^{-3} density averaged from CuCl , 4.14 g cm^{-3} and NaCl , 2.165 g cm^{-3})³⁶.

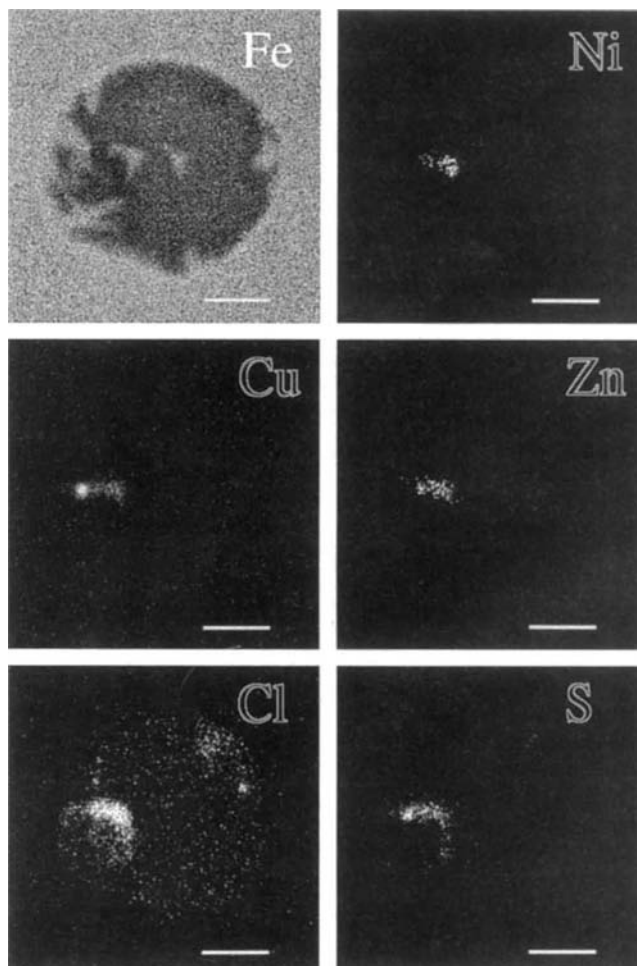


FIG. 3 Element maps of the melt inclusion shown in Fig. 2a, collected by electron microprobe wavelength dispersive spectrometry at conditions of 20 kV accelerating potential, 45 nA beam current, and 600 s accumulating time. The maps of Cl and S were obtained with a PET crystal; Ni, Fe, Zn and Cu with LiF. The scale bar in all maps is $14 \mu\text{m}$. The brightness in the map is proportional to the concentration of an element. These maps demonstrate that the amounts of Cu, Zn, Ni, Cl and S are very low in the silicate glass but are dramatically concentrated in the precipitates on the wall of the bubbles. No physical phases of the precipitates are recognizable, but the element maps suggest that these are sulphides and chlorides. A total of 23 elements were examined but only 6 are shown here. Silver and gold were detected with larger magnifications at conditions of 20 kV accelerating potential, 100 nA beam current and $1,000 \text{ s}$ accumulating time (K.Y., unpublished results). Lead was not detected at such conditions. Similar element maps were obtained of the precipitates within CO_2 -bubbles in the matrix glass of the volcanic rocks (K.Y., unpublished results).

fluids³⁵. Perhaps Ni is precipitated at depth instead of being added to the hydrothermal system. Whether metal-rich magmatic fluids may be transferred to a hydrothermal system remains unresolved; the possible mechanism of such a process is also unclear. □

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CORRESPONDENCE should be addressed to S.D.S. (e-mail: scottsd@ecf.utoronto.ca).

An abiotic model for stromatolite morphogenesis

John P. Grotzinger & Daniel H. Rothman

Department of Earth, Atmospheric and Planetary Sciences,
Massachusetts Institute of Technology, Cambridge,
Massachusetts 02139, USA

STROMATOLITES are laminated, accretionary structures, which are commonly regarded to have formed by the sediment-binding or precipitating activities of ancient microbial mats or biofilms (composed mainly of cyanobacteria), possibly supplemented by abiotic surface precipitation^{1–4}. Stromatolites are thus considered to be a proxy for early life on Earth, as the record of these structures extends back to 3.5 Gyr ago⁵. But as stromatolites only rarely contain fossil microbes, their biogenicity is tacitly assumed on the basis of morphological comparisons with modern, demonstrably biological, structures⁶. Little is known about the physical, chemical and biological processes that

controlled the growth of ancient stromatolites⁴ and, with pioneering exceptions^{7–9}, the analysis of the inherent geometric characteristics of the structures has not been pursued. Here we present a morphological characterization of ancient stromatolites that have growth surfaces with self-affine fractal geometry. We deduce, from both the microscopic textures and the fractal dimension, a purely abiotic dynamical model of stromatolite surface growth that combines chemical precipitation on the growing interface, fallout and diffusive rearrangement of suspended sediment, and uncorrelated random noise. This result calls into question the assumption that organisms—even if present—necessarily played an essential role in determining stromatolite morphology during times when precipitation at the sea floor was common, such as the earlier Precambrian.

The stromatolites analysed in this study form part of a 1.9-Gyr-old subtidal reef developed within the foreland basin of Wopmay orogen, northwestern Canada^{10,11}. The reef is part of the shallowing-upward Cowles Lake Formation in which deep basinal limestone rhythmites and siliciclastic turbidites are overlain by prograding slope facies, capped by reef and back-reef intraclast/ooid grainstone. The reef is 20 metres thick and primarily consists of peak-shaped stromatolites, although domal stromatolites also are locally abundant¹¹. Stromatolite growth surfaces are expressed as laminae which are remarkably irregular, forming a hierarchy of peaks of differing size, and separated by U-shaped depressions (Fig. 1). A high degree of surface roughness is expressed across many different length scales. Three different lamina types are present: (1) laminae, often dark, that preferentially thicken into the depressions; (2) lighter laminae that are uniformly thick in the direction normal to the growth surface; and (3) light laminae that form millimetre-scale irregular projections (Fig. 1a). All laminae consist of fine, microsparitic calcite, with crystal sizes generally in the range of 5–10 µm; darker laminae may contain several per cent clay and silt-sized siliciclastic detritus.

Photographs were taken of the peak-shaped stromatolites exposed along outcrop faces oriented normal to the growth direction (Fig. 1b), and representative samples were collected, cut normal to the growth direction, and polished (Fig. 1a) for detailed analysis. Stromatolitic growth laminae revealed in the polished slab and in the field photograph were traced and digitized for values of height h_j at horizontal locations x_j , $j = 1, \dots, N$, at uniform intervals Δx . If the lamina failed to be single-valued at some x_j , then the average height of that lamina at that x_j was chosen instead. Eleven growth laminae of horizontal length $L = 0.065$ m were obtained (with $N = 64$ and $\Delta x = 1.0$ mm) from the polished slab. Seventeen growth laminae of length $L = 1.20$ m (with $N = 128$ and $\Delta x = 9.4$ mm) were obtained from the field photograph of the outcrop. Each digitized lamina then was multiplied by a Hanning (cosine taper) window function¹² and its power spectrum

$$S(k) = \frac{L}{2\pi} \left| \frac{1}{N} \sum_{j=0}^{N-1} h_j e^{i2\pi jk/N} \right|^2, \quad k = 0, \dots, N-1 \quad (1)$$

was calculated. The average power spectra $\langle S(k) \rangle$ for the polished slab and field photograph, respectively, are shown in Fig. 2. Without adjusting any prefactors, both power spectra obey the same power law $\langle S(k) \rangle = Ak^{-\beta}$, where A is a constant and the exponent $\beta = 2.01 \pm 0.03$ was obtained from linear regression. The two power spectra collectively span nearly three orders of magnitude in wavenumber, with wavelengths ranging from 0.002 to 1.2 m.

The power-law decay of $\langle S(k) \rangle$ shows that the stromatolite laminae have self-affine fractal geometry, where the fractal dimension D may be obtained¹³ from the relation $D = 1 + (5 - \beta)/2$. We thus find $D = 2.50 \pm 0.02$. Having found this one-parameter characterization of stromatolite morphology, we wish to use it to deduce the dynamics of stromatolite growth.

We propose the following mechanisms for the growth of stromatolites in general, for which our example is a particular

case. These mechanisms are: (1) fallout of suspended sediment; (2) diffusive smoothing of the settled sediment (that is, sediment moves downhill at a rate proportional to slope) and surface tension effects in chemical precipitation; (3) surface-normal precipitation; and (4) uncorrelated random noise representative of surface heterogeneity and environmental fluctuations. Viewing these processes macroscopically, that is, in terms of a continuum partial-differential equation, we may express these growth mechanisms as

$$\frac{\partial h}{\partial t} = v_s + \kappa \nabla^2 h + v_p \sqrt{1 + (\nabla h)^2} + \eta(x, t) \quad (2)$$

where the four terms on the right-hand side correspond specifically to the four mechanisms listed above, respectively. Thus v_s is the time-averaged rate of upward growth through sediment settling, κ is an effective diffusion coefficient, v_p is the time-averaged rate of surface-normal precipitation, and η is uncorrelated random noise with zero mean and variance η_0^2 . The square-root factor is a geometric correction that acts to project the surface-normal growth along the h -axis. The smoothing represented by the second term of equation (2) is equally representative of surface tension and diffusion. The net effect of these two processes is combined into the single coefficient κ . In the case of our stromatolite in particular, and other stromatolites in general (including convex-upward morphotypes), the effects of surface tension will tend to dominate in laminae formed by precipitation (the light layers in Fig. 1) whereas the effects of diffusion will tend to dominate in layers which have formed by the fallout of suspended sediment (the dark laminae in Fig. 1).

Assuming that $|\nabla h| \ll 1$, we approximate it by $1 + 0.5(\nabla h)^2$. Then, transforming equation (2) to the comoving frame $h' = h - (v_s + v_p)t$ and dropping primes, we obtain the interface evolution equation of Kardar, Parisi and Zhang¹⁴:

$$\frac{\partial h}{\partial t} = \kappa \nabla^2 h + \frac{v_p}{2} (\nabla h)^2 + \eta(x, t) \quad (3)$$

Numerical solutions of equation (3) and related models for which the contribution from surface-normal growth is not small (specifically, when the quantity $\varepsilon = v_p \eta_0 / \kappa^3$ is not insignificant) yield interfaces that evolve to a statistical steady state with fractal dimension $D \approx 2.6$ independent of initial conditions^{15–17}. Assuming that our measurement of $D \approx 2.5$ is not significantly different from the theoretical prediction of $D \approx 2.6$, our theoretical description is consistent with our quantitative measurements.

We note that when $\varepsilon \rightarrow 0$ (that is, when the contribution from the nonlinearity in equation (3) is insignificant), equation (3) predicts $D = 3$ (ref. 18), in clear contradiction to our measurements. Thus the inclusion of surface-normal precipitation in our mechanistic theory appears essential. Surface-normal growth in the case of the stromatolite studied here involves the precipitation of the two, generally lighter types of laminae (Fig. 1a): one which maintains constant thickness independent of local slope, and another which is expressed by growth of irregular projections that generally are covered by the darker sediment. Some of the taller projections, however, are incompletely covered and influence the shape of overlying laminae, resulting in sharp peaks separated by concave-upward depressions. The irregular projections, which occur at a scale smaller than our resolution but which microscopically show no evidence for preservation of filament moulds or other biogenic microtextures, influenced the structure of the stromatolite reef

over a broad range of length scales to generate its characteristic peaked structure. Thus they constitute the building blocks of an iterative process. Although most other stromatolites produce convex-upward, dome-shaped laminae, our four generic growth mechanisms should still be present. Although our theoretical description has been justified by the use of only one parameter—the fractal dimension D —we note that further confirmation could come from measurement of the time-dependent roughening of initially flat surfaces¹⁴. Thus, future studies should concentrate on the surfaces upon which stromatolite growth is initiated and the way that lamina shape changes upward.

We note that the mechanisms invoked in equation (3) are derived from examination of the stromatolite itself and the quantitative measurement of our stromatolite approximately fits the prediction of a theory. Significantly, the deterministic form of equation (3) describes the evolution of interfaces which are qualitatively similar to growth forms of many stromatolites with more typical, convex-upward morphology (see Fig. 1 of ref. 14 and compare with the image on page 8 of ref. 3). Thus, we consider that we have developed a model which not only approximately fits a measured exponent, but also conforms to physically plausible mechanisms derived from the observations of the stromatolite growth textures themselves.

Our result demonstrates that the morphology of at least some, and perhaps many, types of stromatolites may be accounted for exclusively by abiotic mechanisms, particularly where growth by precipitation is thought to be important. However, inasmuch as microorganisms exist in virtually all shallow marine environments

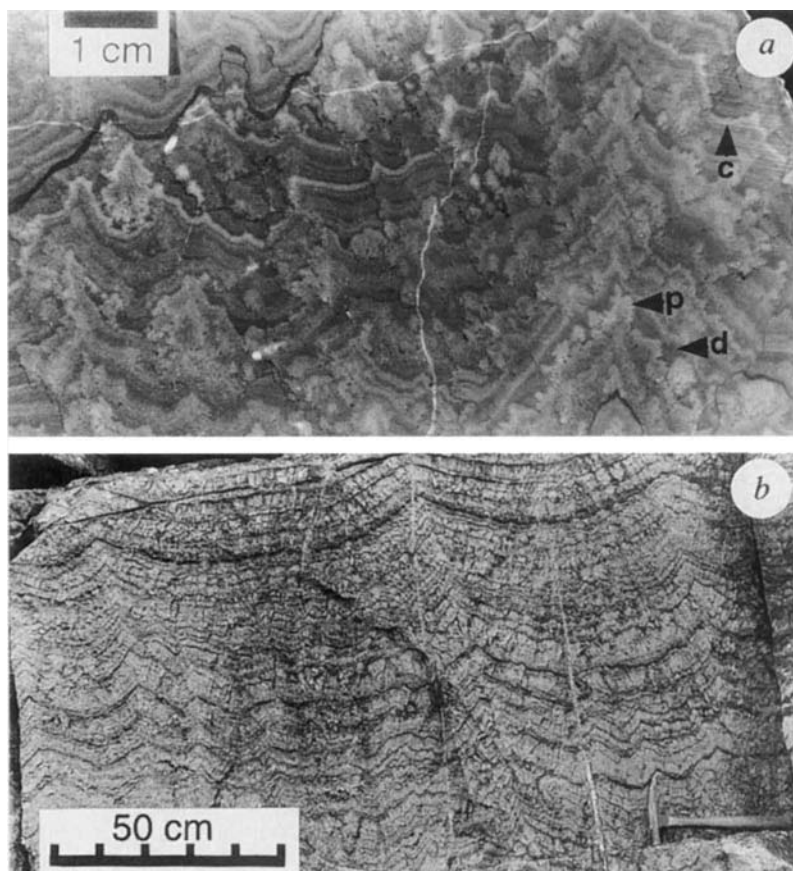


FIG. 1 Peaked-shaped stromatolites from the Cowles Lake Formation reef complex. *a*, Polished slab. Stromatolitic lamination is represented by three types (arrowed) which: thicken into depressions and tend to be darker coloured (*d*); form lighter layers of constant thickness as measured normal to the growth surface (*c*); form lighter layers with millimetre-scale, irregular projections that grow normal to the growth surface (*p*). Note that the millimetre-scale irregular projections are the source of the peaked structure at all larger scales. *b*, Outcrop photograph showing peaks at intermediate and largest scales.

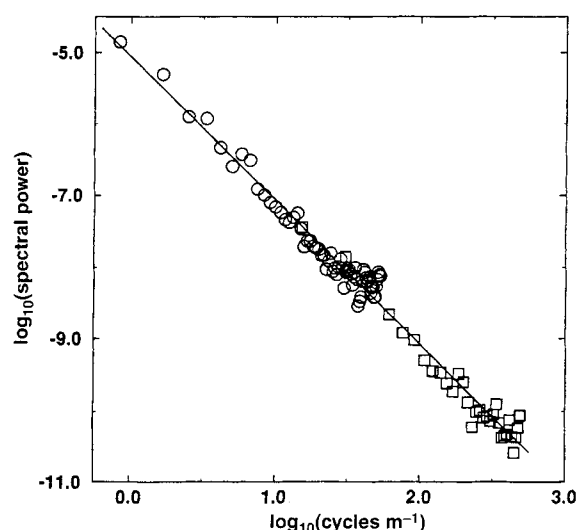


FIG. 2 Plot of $\log_{10}S(k)$ versus $\log_{10}k$, for both the field photograph (circles) and polished slab (squares), compared to the best-fitting line from linear regression (straight line). Both sets of data fit the relation $S(k) \approx k^{-\beta}$ where $\beta = 2.01 \pm 0.03$. The power-law decay of $S(k)$ shows that no single wavenumber component dominates the power spectrum and that the geometry of the stromatolite laminae is statistically scale invariant.

today, it would be misleading to assume that they did not inhabit the surfaces of most, if not all, Precambrian stromatolites. But if they did, then it is no longer clear what role they played in morphogenesis. As the textural evidence for precipitation in stromatolite growth continues to increase^{19–23}, particularly for early Precambrian time when stromatolites are regarded as an important proxy for life on Earth it seems prudent to remain cautious in the assumption of biogenicity without direct evidence supplied by the presence of fossilized microbes that can be shown to have influenced morphogenesis (that is, were not passively residing on a convenient substrate). In attempting to demonstrate through rigorous analysis that morphology may uniquely reflect biology, we emphasize that the null hypothesis—that morphology is the result of physical and chemical processes—cannot be falsified in the case studied here and probably for many other stromatolites. □

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CORRESPONDENCE should be addressed to J.P.G. (e-mail: grotz@grabau.mit.edu).

Evidence for a sound movement area in the human cerebral cortex

Timothy D. Griffiths^{*†‡}, Adrian Rees^{*},
Caroline Witton^{*}, Ra'ad A. Shakir[§], G. Bruce Henning^{||}
& Gary G. R. Green^{*}

Departments of ^{*} Physiological Sciences and [†] Clinical Neuroscience, Newcastle University Medical School, Newcastle upon Tyne NE2 4HH, UK
[‡] Wellcome Department of Cognitive Neurology, Institute of Neurology, 12 Queen Square, London WC1N 3BG, UK
[§] Regional Neurosciences Centre, Charing Cross Hospital, Fulham Palace Road, London W6 8RF, UK
^{||} Department of Experimental Psychology, Oxford University, South Parks Road, Oxford OX1 3UD, UK

HUMAN listeners can localize sounds by the difference in both arrival time (phase) and loudness between the two ears¹. Movement of the sound source modulates these cues, and responses to moving sounds have been detected in animals in primary auditory cortex^{2,3} and in humans in other cortical areas⁴. Here we show that detection of changes in the interaural phase or amplitude difference occurs through a mechanism distinct from that used to detect changes in one ear alone. Moreover, a patient with a right hemisphere stroke is unable to detect sound movement, regardless of whether it is defined by phase or by loudness cues. We propose that this deficit reflects damage to a distinct cortical area, outside the classical auditory areas, that is specialized for the detection of sound motion. The deficit is analogous to cerebral akinopsia (motion blindness) in the visual system, and so the auditory system may, like the visual system⁵, show localization of specialized functions to different cortical regions.

Detection of a sound movement from phase cues was investigated in control subjects by using a binaurally presented tone of 500 Hz in which the phase was sinusoidally modulated at 2 Hz. In one condition, as the phase was advanced at one ear, it was retarded at the other. This produces a changing delay between the waveforms at the ears, or interaural phase modulation (IPM), like that produced by a sound source oscillating in a horizontal arc around the head. Apart from changes in the interaural phase between the ears, this stimulus also modulates the instantaneous phase or frequency at either ear alone; a control stimulus was therefore used in which these frequency changes were identical at the two ears (binaural frequency modulation, FM). The interaural phase difference of the FM stimulus was always zero.

The detection of frequency- and phase-modulated stimuli is shown in Fig. 1a, c for six control subjects. The mean threshold (expressed as modulation index) was 1.24 for the detection of FM, and 0.313 for IPM. The IPM threshold for controls corresponds to a maximum interaural phase delay of 36 deg and, for an environmental sound, an average angular velocity of 85 deg s⁻¹. The data for naive subjects shows inter-subject variation, but the FM threshold for individual subjects exceeds the IPM threshold 4.7-fold (95% confidence interval, 2.8–6.8). Thus control subjects find it easier to detect a phase modulation at the two ears associated with the lateral movement of a sound source than to detect the associated frequency modulation. Together with the subjects' reports of a clear difference in the percept at threshold, this shows that the detection mechanism for IPM is distinct from those previously described for FM at low modulation rates^{6,7}. At higher modulation rates, above 10 Hz, the detection of IPM and FM is similar for all subjects, and the perception of sound movement is lost.

Detection of sound movement from amplitude cues was also investigated in control subjects by using a binaurally presented tone sinusoidally amplitude modulated at 2 Hz. Analogous to the case with phase modulation, the stimuli had amplitude changes

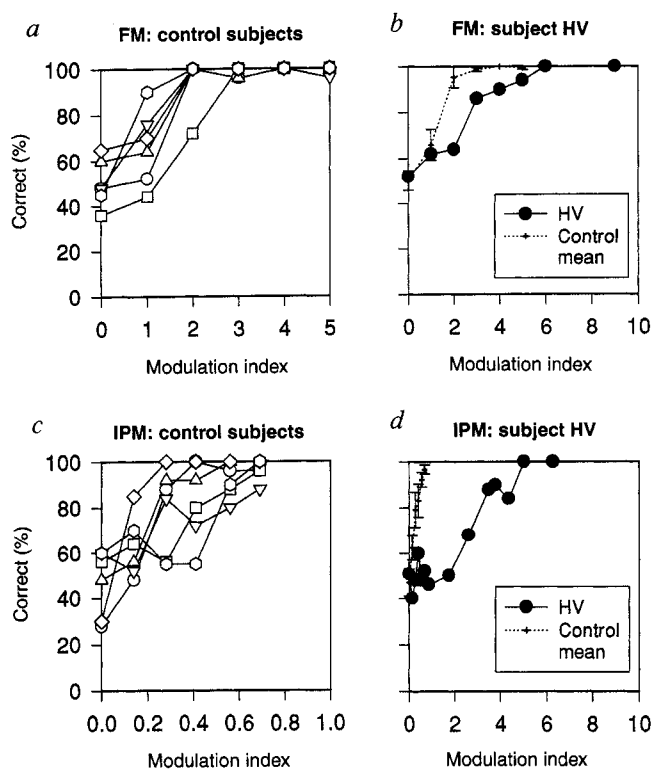


FIG. 1 Frequency and phase-modulation detection. Psychometric functions for 6 naive control subjects (3 male and 3 female, mean age 60 years, mixture of normal subjects and patients with peripheral nervous disorders; open symbols in *a, c*) and subject HV (filled symbols in *b, d*). The mean and standard error of the control data are shown on the graphs for HV. *a, b*, Detection of binaural sinusoidal frequency modulation (FM) plotted as percentage of correct responses against modulation index. *c, d*, Detection of interaural sinusoidal phase modulation (IPM). Modulation rate, 2 Hz. Note that the scales are different in *a* and *c*.

that were either in phase at the ears (binaural amplitude modulation, AM), or out-of-phase at the two ears (interaural amplitude modulation, IAM). The out-of-phase modulation is like that produced by sound moving in an arc around the head. The threshold for the detection of both stimuli by control subjects is similar (threshold modulation index 0.05–0.08) when the stimuli were discriminated from a pure (unmodulated) tone. Above threshold, however, unlike the AM stimulus where the percept remains stationary, the IAM stimulus produces a perception of movement. Moreover, subjects could distinguish the AM from the IAM stimulus (Fig. 2*a*).

Both sets of tests were performed on subject HV, a 75-year-old male who suffered an infarction between the right middle and posterior cerebral artery territories (Fig. 3) one year before testing. He developed a homonymous hemianopia and a mild left hemiparesis that recovered within 1 month of the infarct. Left visual and tactile hemi-inattention, however, persisted after the event.

HV had a deficit in fixed lateralization, although he could indicate reliably the side of a monaural stimulus, and reported a fused sound image for binaural stimuli. His threshold for detecting a fixed-interaural amplitude difference was 5.9 dB at 500 Hz for an apparent sound displacement to the right, and 6.8 to the left, a level at which trained normal listeners, whose thresholds are about 1 dB, report approximately half of the maximum possible lateral displacement⁸. The phase-difference threshold was 82 deg at 500 Hz for apparent sound displacement to the right, below the value producing maximum lateral displacement in normal listen-

ers⁸. A phase-difference threshold could not be demonstrated for apparent displacement to the left.

Tests for the detection of auditory movement cues revealed a striking deficit. When listening to the IPM stimulus at 2 Hz, HV, in contrast to the control subjects, did not perceive sound movement. Further, in contrast to control subjects, his threshold modulation indices for IPM and FM detection were 3.04 and 2.40, respectively (Fig. 1*b, d*). The value for the IPM task corresponds to a maximal interaural phase difference of 348 deg, compared with 36 deg for control subjects. His performance with the IPM and FM tasks was consistent with his detecting both stimuli by their frequency modulation. For both stimuli he reported detection when the "pitch of the sounds wobbled". HV was also unable to distinguish between IPM and FM at the lower modulation rates of 1 Hz and 0.5 Hz. He had similar IPM and FM thresholds (11.7 and 10.10, respectively) at 1 Hz. At the higher rate of 40 Hz, his FM threshold modulation index was 0.56, and, like control subjects, he heard no movement with IPM at this rate.

Like the control subjects using a pure-tone comparison, HV had similar threshold modulation depths for detecting IAM and AM at a modulation rate of 2 Hz (threshold modulation indices 0.27 and 0.23, respectively). Unlike controls, however, he could not distinguish IAM from AM (Fig. 2*b*), nor did he perceive sound movement with IAM, even when the modulation index was 1.0, corresponding to the maximal possible excursion between alternate monaural presentations at each ear. He also failed to distinguish the movement cue with amplitude modulation frequencies of 1 Hz and 0.5 Hz.

Ramp stimuli, in which interaural amplitude and phase differences were varied, were also used. Such stimuli occur when a sound source moves in one direction around the head. HV was unable to perceive movement from large swings in the phase of a tone of 500 Hz (180 deg over 800 ms), or from similarly large amplitude swings, stimuli that normally produce a strong sensation of movement.

HV's ability to detect matched frequency and amplitude changes at the two ears argues strongly against there being a nonspecific deficit in psychophysical performance. A peripheral deficit in the coding of the sound wave by phase locking could produce a deficit in motion detection based on interaural phase information. However, the additional observation of a failure to detect sound movement generated by changes in interaural amplitude differences is consistent with the presence of a central deficit. Although HV had a fixed-lateralization deficit, his ability to perceive binaural stimuli as fused auditory images argues against a deficit in attention to one ear being responsible for the

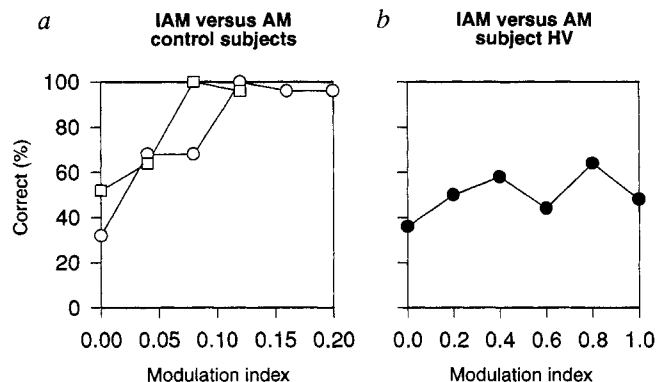
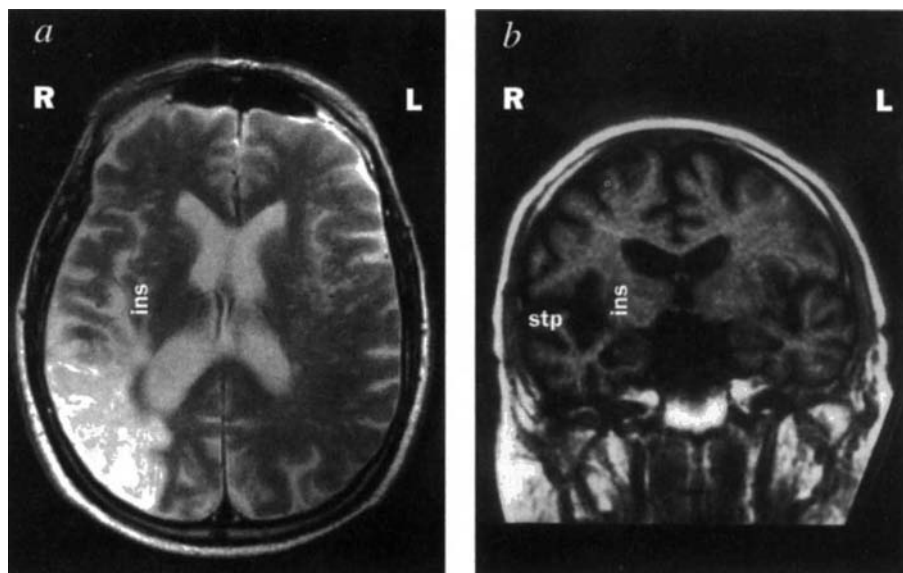


FIG. 2 Discrimination of interaural (IAM) from binaural amplitude modulation (AM). Psychometric functions for *a*, 2 naive control subjects (females aged 59 and 73 years, open symbols), and *b*, subject HV (filled symbols). IAM and AM were presented in separate intervals, and the subject was required to identify which interval contained the IAM. Modulation rate, 2 Hz. Note the difference in scale.

FIG. 3 Magnetic resonance imaging (MRI) scans of patient HV carried out a, 4 months, and b, 12 months after the event. a, A T2-weighted axial section showing an infarction between the middle and posterior cerebral artery territories involving the temporal and parietal cortices. b, A coronal inversion recovery image showing mild right temporal lobe atrophy and involvement of the right insula in the lesion. Ins, insula; stp, superior temporal planum. HV is right-handed with a National Adult Reading Test score consistent with a premorbid IQ of 122. Tests of left-hemisphere function including Western Aphasia Battery were normal, while testing of right hemisphere function revealed moderate impairment of object and space perception. A pure-tone audiogram revealed a symmetrical loss of 20 dB up to 4 kHz.



deficit observed. Unlike patients with the visual motion deficit akinopsia^{9,10}, HV did not report a deficit in the detection of movement of environmental stimuli. This might be explained by his use of other modalities, the availability of other cues, including pinna effects, as well as his sheltered auditory environment.

Our results demonstrate a dissociated deficit in the detection of interaural-phase and interaural-amplitude modulation, with preserved frequency and amplitude modulation detection, and provide support for distinct auditory spatial and temporal processing in humans. Specifically, we have shown there to be a complete deficit in the use of two important cues for the detection of auditory motion using interaural waveform differences, even though the detection of monaural spectral and intensity cues also used for movement detection¹¹ may be preserved. There is debate about the psychophysical mechanism of auditory motion detection, which could be subserved by activation of a sequence of spatial detectors¹² or by velocity detectors¹³. HV has a deficit in fixed auditory lateralization, suggesting the former mechanism. However, he is unable to detect movement produced by a maximum-amplitude excursion between the ears which he could reliably detect for stationary sound, and for phase excursions above his fixed phase threshold for apparent sound displacement to the right. This suggests that there is a dissociation between the fixed lateralization and sound movement detection deficits. The presence of the dissociation at very low modulation rates suggests that it is not a consequence of movement on 'integration time' for fixed spatial performance¹⁴.

We considered whether involvement of classical primary auditory areas or adjacent cortical areas might cause the observed deficit in detection of sound-movement cues. In humans the primary auditory area, A1, lies in the superior temporal planum¹⁵, and is activated during FM tasks¹⁶. Studies using animals have demonstrated that some neurons in A1 are preferentially activated by FM¹⁷ and by moving sounds^{2,3}, and lesions involving the auditory cortex produce deficient behavioural responses to moving sound¹⁸. The right superior temporal planum is affected in HV, although only by mild regional atrophy.

We suggest that HV has a movement-detection deficit caused by lesioning of an area outside A1. The posterior parietal cortex, which is involved in spatial analysis in visual and auditory modalities in the monkey¹⁹, is affected by his lesion. There is evidence for sound activation in humans in this area²⁰, and human lesion studies show a deficit in fixed auditory spatial analysis resulting from right parietal lesions²¹. The infarct also involves the right insula, consistent with a positron emission tomography (PET)

study in normal individuals showing activation of this area during a task requiring attention to changing interaural phase⁴. Both areas are multimodal, and would be rather different from the cortex specialized for visual motion detection in the antero-lateral occipital lobe⁵. □

Methods

Modulation detection was measured using a two-alternative forced-choice technique. Sounds were presented over headphones at a carrier frequency of 500 Hz and sensation level of 60 dB. For the FM, IPM and AM sounds, subjects were required to detect which of two intervals contained the modulated sound with a pure tone in the other interval. The interval in which the sound was modulated and the modulation depth were varied randomly from trial to trial. For the IAM discrimination task, the subject was required to distinguish IAM from AM, a task normal subjects perform by using lateralized movement as a cue. Weibull analysis was performed²² to fit psychometric functions and estimate threshold, defined as the modulation index corresponding to 75% correct detection. (At a fixed modulation frequency, modulation index is proportional to the depth of modulation.) Each point on the graphs corresponds to at least 50 trials for HV and 25 trials for the controls.

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CORRESPONDENCE and requests for materials should be addressed to T.D.G. (t.d.griffiths@ncl.ac.uk).

Disruption of auditory spatial working memory by inactivation of the forebrain archistriatum in barn owls

Eric I. Knudsen & Phyllis F. Knudsen

Department of Neurobiology, Fairchild Science Building, Stanford University School of Medicine, Stanford, California 94305-5401, USA

BARN owls not only localize auditory stimuli with great accuracy, they also remember the locations of auditory stimuli and can use this remembered spatial information to guide their flight and strike¹. Although the mechanisms of sound localization have been studied extensively^{2,3}, the neurobiological basis of auditory spatial memory has not. Here we show that the ability of barn owls to orient their gaze towards and fly to the remembered location of auditory targets is lost during pharmacological inactivation of a small region in the forebrain, the anterior archistriatum. In contrast, archistriatal inactivation has no effect on stimulus-guided responses to auditory targets. The memory-dependent deficit is evident only for acoustic events that occur in the hemifield contralateral to the side that is inactivated. The data demonstrate that in the avian archistriatum, as in the mammalian frontal cortex, there exists a region that is essential for the expression of spatial working memory and that, in the barn owl, this region encodes auditory spatial memory.

The central nervous system continuously stores information about the spatial locations of stimuli in a temporary form referred to as spatial working memory⁴. This form of memory operates over time periods of seconds, retaining spatial information that can be used later to guide behaviours in response to stimuli that are no longer present. Much attention has been paid to the characterization of visual spatial working memory⁵⁻⁷. However, auditory spatial working memory is equally essential, if animals are to build and maintain a representation of their auditory worlds.

We assessed auditory spatial working memory in barn owls by using delayed-response tasks. The first task (Fig. 1a), was a modified version of the oculomotor delayed-response task used commonly to assess visual spatial working memory in primates^{8,9}. Three owls (named TX, PH and DA) were trained to stand on a perch and fix their gaze on a small light-emitting diode (zeroing light). In owls, the direction gaze is indicated by the orientation of the head, as the eyes are essentially stationary in the head. A noise burst was then presented from one of 14 target locations in the horizontal plane in front of the owl (see Methods). In response to the noise burst, the owl turned its head towards the sound target (saccade 1). The sound was turned off immediately after the owl oriented to it; the owl then turned its head back towards the illuminated zeroing light (saccade 2). Usually, a buzzer located at the site of the zeroing light was activated briefly to bring the owl's gaze back to the light. When the zeroing light was turned off (0.1 to 2.0 s after saccade 2 was completed), the owl turned its head (saccade 3) and flew to a landing rail in the location of the previously presented sound to receive a food reward. In this task, saccades 1 and 2 were stimulus-guided. In contrast, saccade 3 and the strike at the rail were guided by remembered spatial information.

Remembering the locations of auditory stimuli may be mediated by the forebrain, and a region in the barn owl's forebrain that contributes to auditory spatial behaviour is the anterior archistriatum¹⁰. This region (Fig. 2a) exhibits many of the anatomical and functional properties of the primate frontal eye fields (FEF) in the frontal cortex¹¹, where neuronal activity has been shown to encode visual spatial working memory^{12,13}. We tested whether neuronal activity in the owl's anterior archistriatum is

essential for the behavioural expression of auditory spatial working memory.

Neurons in the archistriatum were inactivated with small (100–200 nl) injections of muscimol (see Methods). Stainless-steel guide tubes were implanted¹⁰ to direct the injections into the archistriatum on the right side in owl TX and on the right and left sides in owls PH and DA (Fig. 2). Muscimol inactivates neurons for several hours¹⁴, so control (no injection) and experimental data were gathered on alternate days.

The control performance of all three owls on the triple saccade task was similar and is represented by the data from owl DA in Fig. 1b, c. The stimulus-guided saccade (saccade 1) was highly accurate and precise (Fig. 1b). The memory-guided saccade (saccade 3), made 2 to 15 s after the offset of the auditory stimulus, was less precise than the stimulus-guided saccade, as indicated by the increase in the dispersal of dots in Fig. 1c compared with that in Fig. 1b. Flights to the landing rail exhibited about the same precision as the memory-guided saccades.

Unilateral injections into the archistriatum disrupted memory-guided behaviours for targets located on the contralateral side (Fig. 1d–f, shaded side). Stimulus-guided saccades (saccade 1) to all target locations were made with normal accuracy (open circles in Fig. 1d–f; general linear test, $P > 0.05$). In contrast, memory-guided saccades and strikes made in response to contralateral stimuli either did not occur (data points along the abscissa and histograms in Fig. 1d–f) or were uncorrelated ($P > 0.22$) with the location of the auditory target. For injection sites TX right (right archistriatum of owl TX; Fig. 1d) and PH right (data not shown), the owls made saccade 3 in less than half of the trials with contralateral stimuli, and when saccade 3 was made, it was to a random location on the wrong side (Fig. 1, legend). For the other three injection sites (for example, Fig. 1e, f), the owls either did not turn at all after the offset of the zeroing stimulus or they turned randomly to the left or right. The inability to direct gaze towards remembered targets on the contralateral side cannot be explained by either a sensory or a motor deficit, as the owls oriented accurately towards the target at the beginning of every trial (saccade 1). Instead, the memory of the stimulus itself or the ability of memory to direct attention or gaze to contralateral targets must have been disrupted.

Memory-guided saccades (saccade 3) made in response to ipsilateral stimuli were not significantly affected (general linear test, $P > 0.05$) by the injection (filled circles on the unshaded side in Fig. 1d–f). However, strikes towards ipsilateral stimuli were affected for three of the five injection sites (for example, crosses on the unshaded side in Fig. 1d–f). Nevertheless, in all cases, memory-guided saccades and strikes to ipsilateral stimuli were made in every trial, and the end points of the saccades and strikes correlated ($P < 0.05$) with the location of the auditory target.

A second behavioural task that required memory to guide only the flight and strike also revealed a severe contralateral deficit. The owls were trained simply to turn the head (saccade 1; Fig. 3a) and fly to the source of a 100-ms noise burst. The head saccade was stimulus-guided. However, because the auditory stimulus was over before the saccade was completed, the animal had to remember the stimulus location to fly and strike. The length of time that a memory of the stimulus needed to be maintained to initiate flight was less than 1 s.

Under control conditions, the owls performed this behaviour reliably. In contrast, after an injection of muscimol at any of the five sites, the owl often failed to fly at targets that had been presented on the contralateral side, even though it continued to fly and strike consistently at targets that had been presented on the ipsilateral side (Fig. 3b, c). For TX right, the contralateral deficit was total (data points along the abscissa and histogram in Fig. 3b).

To verify that muscimol injections did not interfere with the ability to fly and strike accurately at contralateral stimulus locations, we interspersed short noise-burst trials with trials in which the sound remained on (Fig. 3d). For all locations and all injection sites, the owls turned and flew accurately towards ongoing sounds

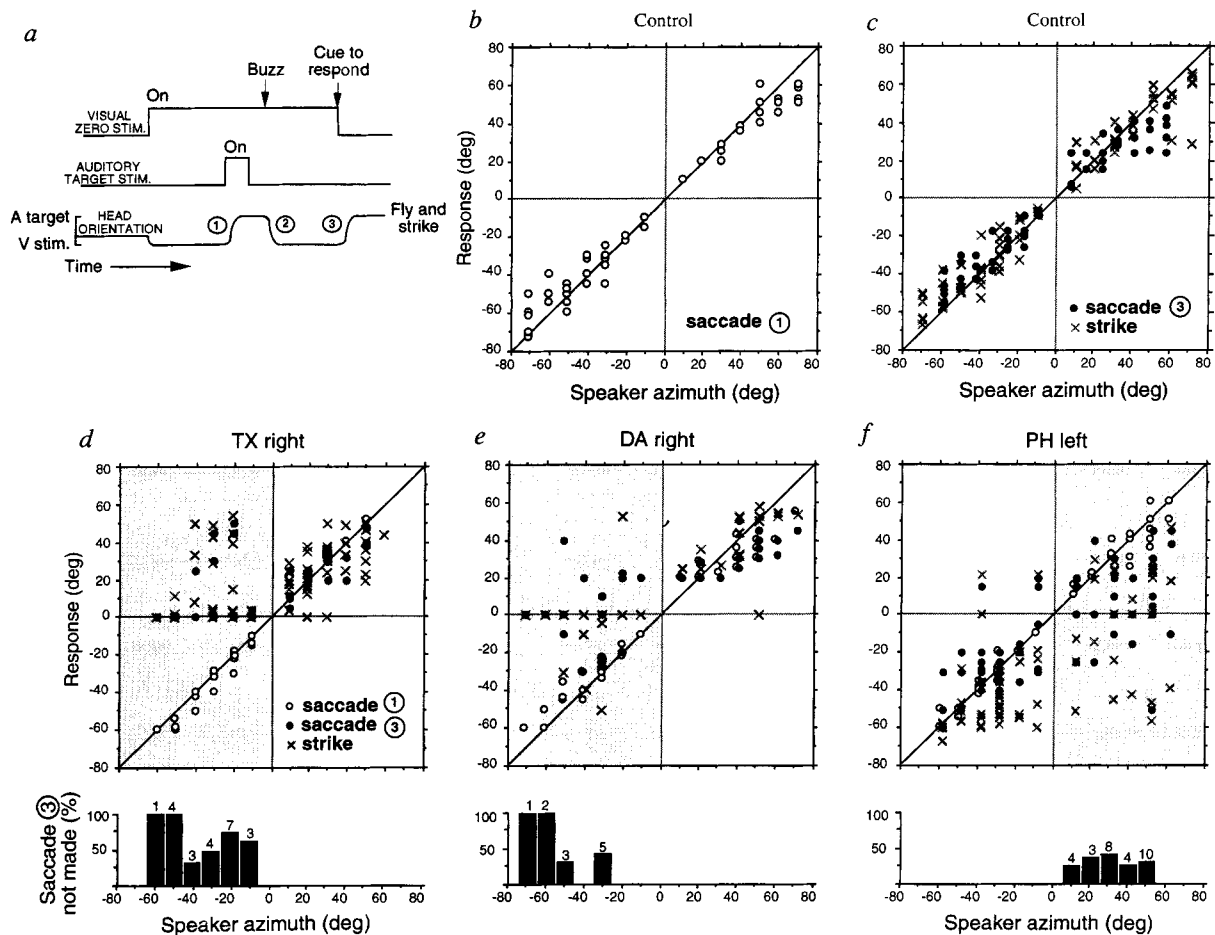
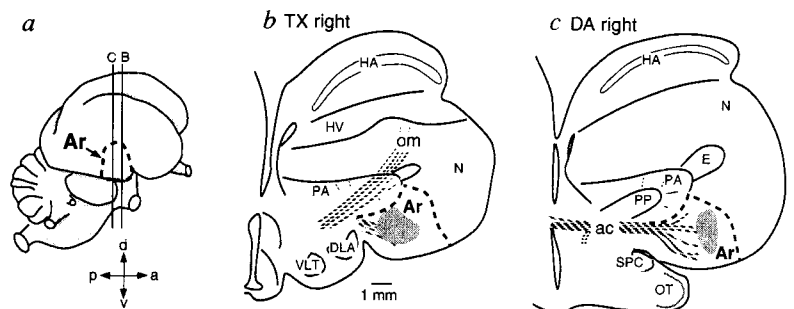


FIG. 1 The triple saccade and strike task done under control conditions and during unilateral inactivation of the anterior archistriatum. *a*, Schematic diagram of the behavioural experiment. Top, the period of illumination of the zeroing light and the occurrence of the zeroing buzz. Middle, the timing of the acoustic target stimulus. Bottom, schematic changes in head orientation (saccades 1, 2 and 3) in response to the visual (V) and auditory (A) stimuli. *b*, The accuracy of stimulus-guided saccade 1 under control conditions for owl DA. Degrees to the right are positive and to the left are negative. The diagonal line represents perfect accuracy. *c*, The accuracy of memory-guided saccade 3 (filled circles) and strikes (crosses) under control conditions for owl DA. Some of the head saccades were not recorded on video tape. This accounts for the trials for which there are data for the strike but not for the head saccade. *d–f*, The accuracy of stimulus-

guided saccade 1 (open circles) and of memory-guided saccade 3 (filled circles) and strikes (crosses) after unilateral injections of muscimol at sites TX right, DA right and PH left, respectively. The side contralateral to the injection is shaded. Points along the abscissa (response = 0°) represent trials in which either saccade 3 or a strike was not made within 60 s of the cue to respond (offset of the zeroing stimulus). For TX right (*d*) and PH right (data not shown), strikes were made consistently to the wrong side when the stimulus was located on the contralateral side. This suggests the effects of contralateral neglect. Histograms indicate the percentage of trials in which the owl failed to make saccade 3, the memory-guided saccade. The number above each bar indicates the number of trials at that location. In these trials, the owl continued to fix its gaze on the location of the zeroing light for 60 s after the light was turned off.

FIG. 2 Muscimol injection sites in the anterior archistriatum. *a*, Side view of the brain of a barn owl, showing the location of the archistriatum (Ar; dashed border). Vertical lines indicate the levels of the most anterior and most posterior of the archistriatal injection sites. *b*, A reconstruction of the most anterior of the injection sites (TX right). Injection sites were reconstructed on the basis of the distribution of rhodamine-conjugated latex microspheres (200 nl) injected at each site at the end of the experiments. *c*, A reconstruction of the most posterior of the injection sites (DA right). All of the injection sites were centred in the anterior archistriatum within a 1.0-mm zone extending from the level of the anterior commissure (ac) to the level of the occipitomesencephalic tract (om). Abbreviations: a, anterior; p, posterior; d, dorsal; v, ventral; Ar, archistriatum; DLA, nucleus dorsolateralis anterior thalami; E, ectostriatum; HA, hyperstriatum accessorium; HV, hyperstriatum ventrale; N, neostriatum; PA, paleostria-



tum augmentatum; PP, paleostriatum primitivum; SPC, nucleus superficialis parvocellularis; VLT, nucleus ventrolateralis thalami; OT, optic tectum.

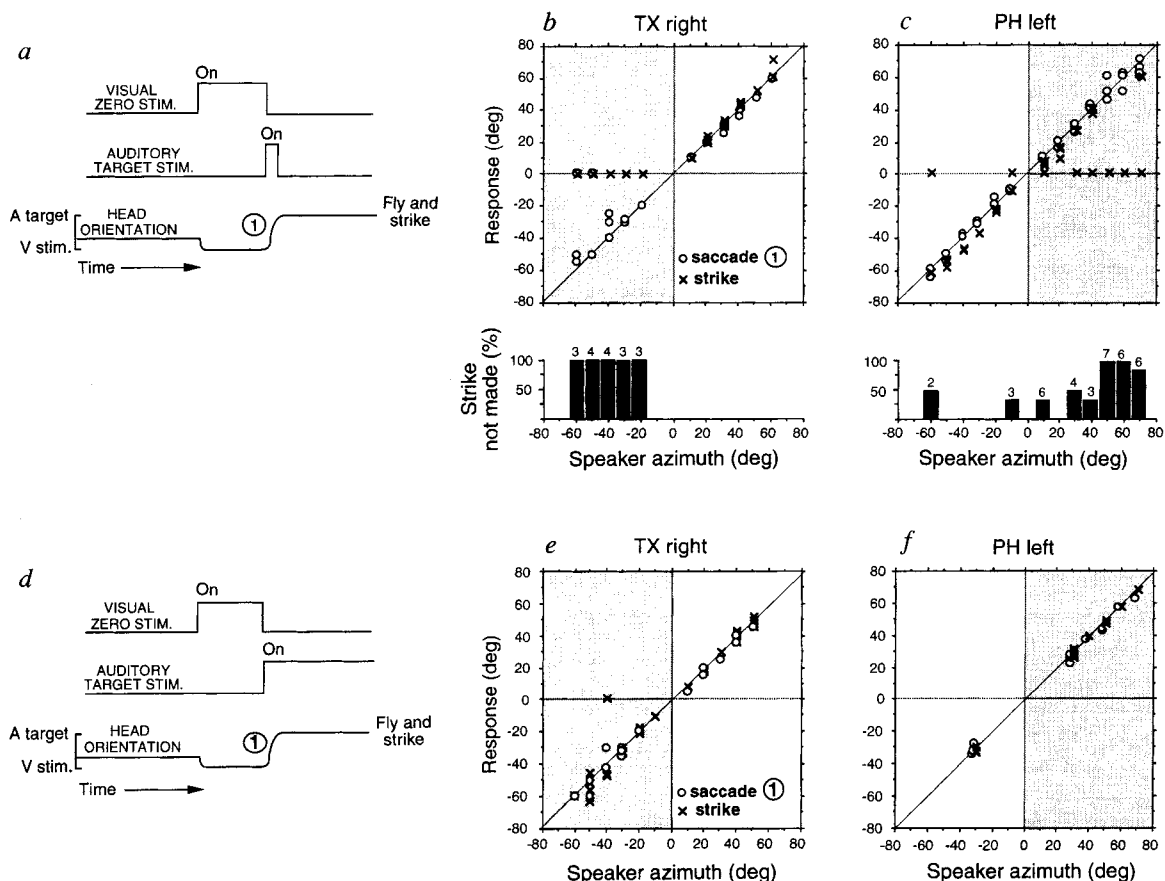


FIG. 3 Single saccade and strike tasks done during unilateral inactivation of the anterior archistriatum. *a*, Schematic diagram of the behavioural procedure using a short-duration auditory target stimulus. Top, visual zeroing stimulus. Middle, 100-ms noise burst. Bottom, schematic changes in head orientation in response to the visual (V) and auditory (A) stimuli. The auditory stimulus was the cue to respond. (*b*, *c*). The accuracy of stimulus-guided saccades (open circles) and of memory-guided strikes (crosses) after unilateral injections of muscimol at sites TX right and PH left, respectively. The side contralateral to the side of the injection is shaded. Degrees to the right are positive and to the left are negative. The diagonal line indicates perfect accuracy. Points along the

abscissa (response = 0 deg) represent trials in which the owl did not fly within 60 s of the end of the auditory stimulus. Histograms indicate the percentage of trials in which the owl did not fly. The number above each bar indicates the number of trials at that location. *d*, Schematic diagram of the single saccade paradigm using a long auditory target stimulus. The auditory target stimulus continued either until the owl flew or for 5 s, whichever came first. *e*, *f*, The accuracy of stimulus-guided saccades (open circles) and stimulus-guided strikes (crosses) after unilateral injections of muscimol at sites TX right and PH left, respectively. The conventions for plotting the data are the same as those described for *b* and *c*.

(crosses in Fig. 3*e*, *f*). To test whether the anterior archistriatum is essential for the initiation of flight, we injected both sides simultaneously in owls PH and DA: after the injections, both owls were still capable of making head saccades, initiating flight and flying normally. The results indicate that a region in the anterior archistriatum is necessary for memory-guided flight and for memory-guided changes in gaze direction, but not for flight or gaze control *per se*.

The anterior archistriatum is the main source of forebrain output to the brainstem and spinal cord, analogous in many ways to the mammalian frontal cortex^{11,15}. The data demonstrate that an area in the anterior archistriatum provides a critical link in the pathway that subserves memory guidance of both gaze control and flight. The data also demonstrate that auditory spatial working memory is lateralized in the central nervous system, with each archistriatum representing the memory of contralateral auditory targets. Visual spatial working memory is lateralized in the same way in the FEF and dorsolateral prefrontal cortex of monkeys^{5,7,8,12,13}. Finally, the pathways through the midbrain and forebrain that mediate sound localization behaviour^{10,16} are incapable of maintaining information about the location of auditory stimuli without the contribution of the archistriatum. By analogy, the same might be expected of the FEF or dorsolateral prefrontal cortex in primates. In conclusion, the archistriatum is essential for

spatial memory or the capacity to access spatial memory about acoustic events that have occurred in the recent past. □

Methods

Apparatus. Experiments were done in a dimly lit room, the walls of which were lined with acoustic foam and covered with black cloth. The experimental apparatus was a semicircular table top, 120 cm in radius, covered with artificial grass. A perch 8 cm wide was centred along the straight edge, and along the semicircular edge was a 15-cm-high curtain of black cloth. The curtain hung from a landing rail, which was calibrated in 1° increments. The target speaker and a remotely controlled feeder moved on a boom behind the black curtain and were hidden by the curtain. The zeroing light was attached to the curtain at the centre of the semicircle and defined 0° azimuth. A buzzer was attached to the wall of the room just behind the zeroing light. The speaker could be positioned at 10° intervals, up to 80° left or right of the zeroing light. The duration and timing of auditory and visual stimuli were controlled with a programmable microprocessor. The spectrum of the target noise was flat (2 dB) from 2 to 10 kHz. Sound level was varied randomly from trial to trial over a range of 30–50 dB, measured at the perch. Rise and fall times were 0 ms. Small pieces of mouse were presented as a reward from the feeder. A video camera recorded the animal's head saccades from above. A thin white stick, attached to the top of the head and aligned with the beak, indicated the orientation of the head. Head orientations were assessed afterwards by measuring responses on a television screen. Strike accuracy was based on the centre of the talon spread on the rail. Statistical comparisons of the experimental and control data were made by applying the general linear test¹⁷, using $P < 0.05$ as the criterion for significance.

Injections. Muscimol was injected using a 28-Ga stainless-steel injector tube coupled by polyethylene tubing to a 1- μ l Hamilton syringe. The injector tube was inserted into a permanently implanted, 23-Ga guide tube that was targeted on the anterior archistriatum. Muscimol ($5 \mu\text{g } \mu\text{l}^{-1}$) was injected in dosages of $1 \mu\text{g}$ (200 nl) in owls TX and PH and $0.5 \mu\text{g}$ (100 nl) in owl DA. The injections were made 30 s after inserting the injector tube into the brain, over a period of 1 min. The injector tube was removed rapidly and the guide tube was sealed with a rod coated with antibiotic ointment. The extent of neuronal inactivation was not measured in this study; however, injections of this size (0.5 – $1.0 \mu\text{g}$) have been estimated to inactivate tissue 1–2 mm from the site of injection¹⁰.

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CORRESPONDENCE and requests for materials should be addressed to E.I.K. (e-mail: eric@brio.stanford.edu).

Calcium influx and transmitter release in a fast CNS synapse

J. G. G. Borst & B. Sakmann

Abteilung Zellphysiologie, Max-Planck-Institut für medizinische Forschung, Jahnstrasse 29, D-69120 Heidelberg, Germany

CALCIUM entry through presynaptic calcium channels controls the release of neurotransmitter¹. It is not known whether the putative calcium sensor that triggers this rapid neurotransmitter release is close enough to be activated by the large increase in the Ca^{2+} concentration (calcium 'domain') reached within nanometres of a single calcium channel or whether many channels have to open². We tested this in a calyx-type synapse in the rat medial nucleus of the trapezoid body. We compared the quantal content of postsynaptic currents with the presynaptic calcium current that flows during an action potential, and the results suggest that more than 60 calcium channels open for each vesicle that is released. In addition, we dialysed terminals with the slow calcium buffer EGTA, which reduced phasic transmitter release at concentrations as low as 1 mM. These results indicate that the distance that calcium ions must diffuse to reach the calcium sensor is relatively long, and that therefore Ca^{2+} entry through multiple calcium channels is needed to release a vesicle.

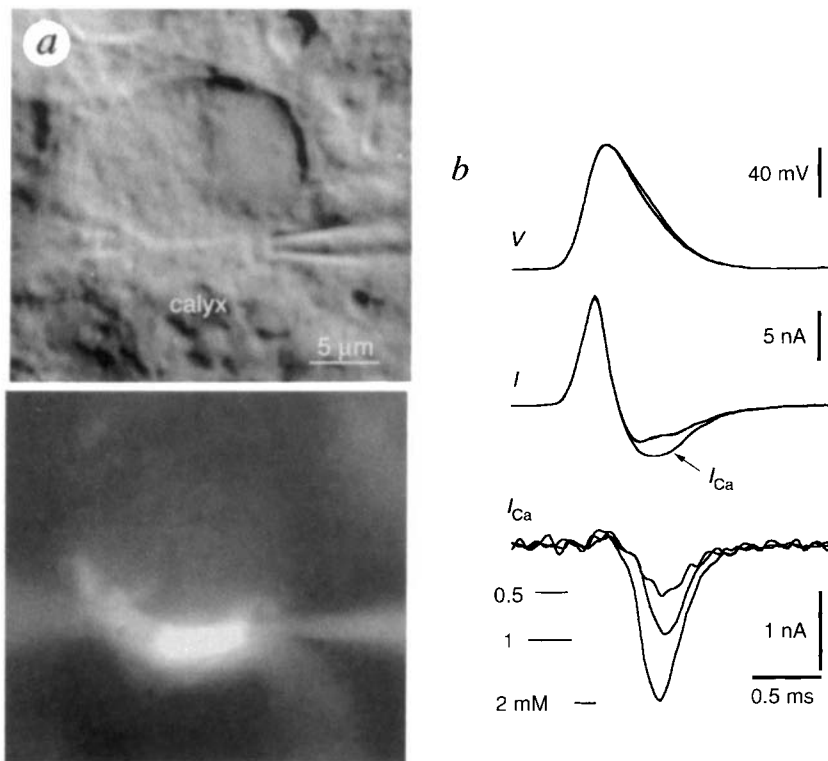
We measured the calcium current that flows into terminals (the calyces of Held) during an action potential by voltage clamping them with an action potential waveform command after blocking sodium and potassium currents³. The calcium current was large and brief (Fig. 1). It was essentially a tail current that activated shortly after the peak of the action potential and ended before repolarization was complete. Peak amplitude was $2.6 \pm 0.2 \text{ nA}$

($103 \pm 9 \text{ pA pF}^{-1}$), and the current integral was $0.89 \pm 0.04 \text{ pC}$ ($n = 12$). Its time course was well described by a gaussian with a standard deviation of $200 \mu\text{s}$. Peak amplitudes, which were reached at around -30 mV , were $42 \pm 7\%$ larger than the peak amplitudes of the current–voltage relations measured in the same experiments, which were reached at around 0 mV . The calcium current was reduced when the extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_0$) was lowered (Figs 1b and 2).

In separate experiments, we compared the sensitivity to $[\text{Ca}^{2+}]_0$ of the calcium currents with that of the synaptic currents. A single action potential was elicited in the presynaptic terminal by stimulation of an afferent fibre in the trapezoid body, which was followed within less than 1 ms by a large ($5.3 \pm 0.4 \text{ nA}$ at -80 mV ; $n = 44$) excitatory postsynaptic current (EPSC)^{4,5}. EPSC amplitudes depended strongly on $[\text{Ca}^{2+}]_0$; the amplitude of the EPSCs was proportional to the fourth power of the presynaptic calcium influx⁶ (Fig. 2). At $0.25 \text{ mM } [\text{Ca}^{2+}]_0$, more than 60% of presynaptic action potentials failed to evoke an EPSC ($n = 19$), and the amplitudes of evoked and spontaneous responses were similar. Therefore, the responses that were evoked probably represent the quantal current¹, that is, the EPSC elicited by the release of glutamate from a single vesicle. Amplitudes of quantal EPSCs were variable (coefficient of variation around 0.5; Fig. 3a), and amplitude histograms were skewed towards larger values, as in other calyx-type synapses^{7,8}. We measured the time course of release at $0.25 \text{ mM } [\text{Ca}^{2+}]_0$ by counting single quanta⁹, and compared it with the time course of the calcium current. Most release events occurred during a time window of 1–2 ms (Fig. 3a, b), very similar to the release from motor nerve terminals⁹. The delay between the average location of the peak of the calcium current ($n = 12$ experiments) and of the delay histogram ($n = 10$, obtained from the fit with the Poisson function) was 0.5 ms (Fig. 3b). The 'jitter' (fluctuation) in the individual releases was not due to a slowing of the time course of release at low $[\text{Ca}^{2+}]_0$, as the averaged EPSC at low $[\text{Ca}^{2+}]_0$ was very similar to the time course at $2 \text{ mM } [\text{Ca}^{2+}]_0$ (Fig. 3c, top). However, quantal EPSCs were much faster than the large, multiquantal EPSCs (Fig. 3c, bottom). Rise times (20–80%) of quantal EPSCs were less than $150 \mu\text{s}$ ($n = 10$), whereas those at $2 \text{ mM } [\text{Ca}^{2+}]_0$ were 200 – $290 \mu\text{s}$ ($n = 10$). This indicates that the difference in the time course of quantal and multiquantal EPSCs is probably caused by the jitter in the release of the individual vesicles^{8,10}. The amplitude of the EPSC at $2 \text{ mM } [\text{Ca}^{2+}]_0$ was 151 ± 14 times the quantal amplitude, which was $43 \pm 5 \text{ pA}$. However, this will be an underestimate of the true quantal content¹¹ because of variability in the latencies of the individual releases. Therefore we also calculated the ratio of the charge of the EPSC at 2 mM and the quantal charge ($83 \pm 11 \text{ fC}$), which yielded a quantal content of 210 ± 22 ($n = 10$). This high quantal content agrees with the large number of active zones observed in electron microscopic studies¹². The many vesicles that are released seem to act independently, suggesting that, despite its much larger quantal content, this synapse acts in a similar way to most CNS synapses¹³.

A comparison of the total calcium influx with the quantal content shows that around 13,000 calcium ions flow in for each vesicle that is released. This value is similar to an estimate obtained in the squid giant synapse^{3,14}, but very different from another calyx-type preparation, the chick ciliary ganglion, where it has been suggested that under certain conditions less than 200 calcium ions are sufficient to cause vesicle release¹⁵. Similarly, we calculate that at least 60 channels open for each vesicle that is released, as the peak of the calcium current was reached at around -30 mV and at this potential less than 0.2 pA flows through a single calcium channel¹⁶. The high number of channels that open for each quantum released are an indication that the domains of multiple calcium channels contribute to the release of a single vesicle. However, not all calcium channels that open necessarily contribute to release⁶. We therefore investigated the effect on synaptic transmission of the slow¹⁷ calcium buffer EGTA, the on-rate of which is 160 times slower than BAPTA at pH 7.2

FIG. 1 A large calcium current flows into presynaptic terminals in the MNTB during an action potential. *a*, Infrared differential interference contrast (IR-DIC) image (top) and Lucifer yellow fluorescence image (bottom) of the calyx with the two recording electrodes and the principal cell, taken at the end of the experiment. *b*, Voltage (*V*) and current (*I*) during two-electrode voltage clamp with an action potential waveform. Two passive responses were obtained by scaling a reduced or a reduced and inverted action-potential waveform; the responses overlay well. The current needed for the full-sized action potential was similar to the passive response during the depolarizing phase of the action potential, but the inward current (middle) was clearly larger during the repolarization. The difference between the passive and the active responses is shown at the bottom (I_{Ca} , 2 mM $[Ca^{2+}]_o$). This current was reduced in 1 or 0.5 mM $[Ca^{2+}]_o$. In *b*, each trace is the average of 6 responses. All data are from the same experiment.



(M. Naraghi and E. Neher, personal communication). We did this by dialysing calyces with different concentrations of EGTA during simultaneous whole-cell recordings of calyces and principal cells⁵, because EGTA has been used as a test for domain overlap in other terminals or cells². After dialysis of the calyx with a solution that contained 1 mM EGTA, the EPSC amplitudes were smaller than in the presence of the endogenous buffer (Fig. 4*a*). The effect of EGTA was concentration dependent (Fig. 4*b*).

Several control experiments were performed (not shown) to address modes of action of EGTA on synaptic transmission other than by buffering the Ca^{2+} that entered during the presynaptic action potential. Dialysis of terminals with a solution containing 16 mM EGTA and 6 mM $CaCl_2$ (free $[Ca^{2+}]$ around 100 nM, close to the resting $[Ca^{2+}]_i$ of terminals¹⁸) reduced EPSCs to $57 \pm 7\%$ ($n = 5$), similar to the effect of a solution that contained only 10 mM EGTA. This suggests that EGTA did not reduce EPSC amplitudes by lowering the resting $[Ca^{2+}]_i$. The relative effect of EGTA on the release from the terminal was very similar at different $[Ca^{2+}]_o$ levels (Fig. 2*a*), arguing against EGTA reducing the calcium sensitivity of the release. EGTA did not lower quantal sizes, or slow the time course of release of individual vesicles,

which would have reduced the peak amplitude of the multiquantal EPSCs. The jitter in the delays of this 'phasic' component of the release was similar in the presence of the endogenous calcium buffer or after dialysis with a high concentration of EGTA. Similar delay histograms of phasic release, containing 40–350 events, were obtained with the endogenous buffer ($n = 4$, Fig. 3), in the presence of 50 μM EGTA ($n = 3$), 1 mM EGTA ($n = 2$), 10 mM EGTA ($n = 1$), or 16 mM EGTA with 6 mM $CaCl_2$ added ($n = 2$). The time constant of decay and the start of the Poisson function used for fitting were similar in intact terminals and after dialysis with a high EGTA concentration (0.18 ± 0.02 versus 0.20 ± 0.04 ms, and 0.85 ± 0.05 versus 0.82 ± 0.01 ms, respectively). This suggests that, even at high concentrations, EGTA does not change the time course of phasic release (defined here as release in the first 3 ms), as has been observed in the crayfish neuromuscular junction¹⁹. However, EGTA blocked the 'delayed release'²⁰, the roughly threefold increase in the probability of release following the end of the phasic release period. The results of these control experiments suggest that EGTA probably acts by binding calcium ions that enter during a presynaptic action potential before they can reach the calcium sensor.

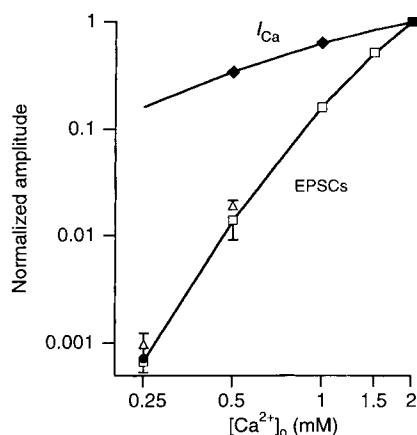


FIG. 2 Dependence of presynaptic Ca^{2+} inflow and postsynaptic currents on external calcium. Amplitude of the presynaptic calcium current (filled diamonds) during an action potential, and of the evoked postsynaptic current at different $[Ca^{2+}]_o$, shown on a double-logarithmic plot. EPSCs were recorded with intact terminals (open squares), or in simultaneous pre- and postsynaptic recordings in which the terminal was dialysed with a low (50 μM, open triangles) or a high (10 mM EGTA or 16 mM EGTA with 6 mM $CaCl_2$ added, filled circles) concentration of EGTA. Amplitudes were normalized to the average amplitudes in 2 mM $[Ca^{2+}]_o$ in the same experiment. In the low- or high-EGTA experiments, the average of responses obtained between 10 and 15 min after presynaptic break-in was taken. The calcium dependency was very similar after dialysis with EGTA-containing solutions. Transmitter release is proportional to the fourth power of the presynaptic calcium inflow; the solid line through the calcium current amplitudes was calculated by raising the line going through the EPSC amplitudes to the one-fourth power. There were at least four experiments for each point.

Our experiments provide a rough estimate of the diffusional distance between calcium channels and the putative release sensor in a nerve terminal in the central nervous system (CNS). Phasic release was still observed in the presence of 10 mM EGTA, and an eightfold reduction in $[Ca^{2+}]_0$ did not slow release, suggesting that the diffusion and binding of calcium ions to the calcium sensor constitutes only a small part of the 0.5 ms delay between the peak of the calcium current and the peak of the release. This limits the distance to which calcium can diffuse to much less than 1 μm , assuming a diffusion constant for Ca^{2+} of $300 \mu\text{m}^2 \text{s}^{-1}$. However, it is unlikely that a vesicle mainly experiences calcium entering through just one calcium channel. Both the large size of the

calcium current during a presynaptic action potential and the reduction of synaptic transmission by a low concentration of EGTA indicate that multiple calcium channels participate in the release of a single vesicle. The absence of a clear effect when high concentrations of EGTA are injected into the rapidly transmitting squid giant synapse²¹ suggests that the diffusional distance for calcium to the release sensor in that synapse is just a few nanometres, too short a distance for a slow calcium buffer such as EGTA to intercept calcium ions before they bind to the putative calcium sensor². In contrast, in chromaffin cells²² or pituitary nerve terminals²³, EGTA reduces release at millimolar concentrations, in accord with the diffusional distance being much larger in these cells². Although EGTA will be able to bind some calcium ions even in the direct vicinity of calcium domains²⁴, our observation that, in the medial nucleus of the trapezoid body (MNTB), 1 mM EGTA gave a substantial reduction of synaptic currents argues against a model for fast synaptic transmitter release in which a vesicle primarily experiences the domain of high $[Ca^{2+}]$ arising from the close proximity of a single calcium channel². At larger distances, the calcium concentrations will rapidly decrease²⁴.

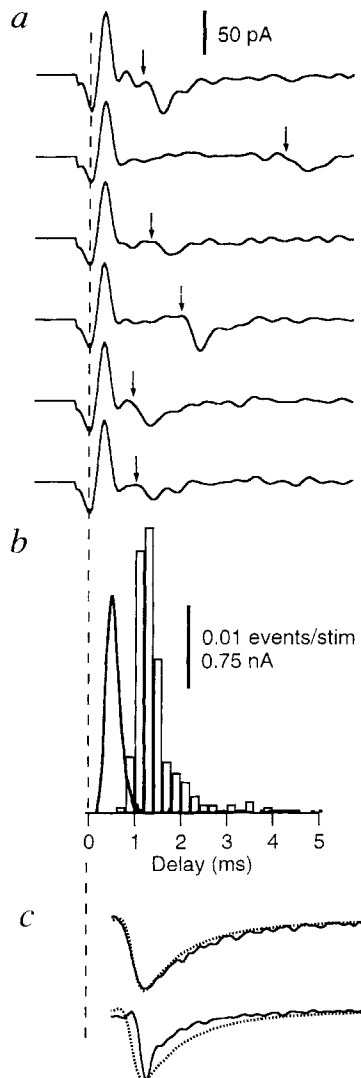


FIG. 3 Time course of quantal release. *a*, Quantal EPSCs at a $[Ca^{2+}]_0$ of 0.25 mM. Stimulation artefact (at beginning of trace) has been blanked. Delays were calculated from the negative peak of the prespike (broken line) to the beginning of the EPSCs (arrows), which followed from the pattern detection algorithm. *b*, Delay histogram, constructed from responses to around 2,800 stimuli. The time course of the calcium current (solid line, from Fig. 1*b*) is shown for comparison. *c*, Comparison of the time course of EPSCs at 0.25 and 2 mM $[Ca^{2+}]_0$ (same experiment and same time scale as *a* and *b*). Solid traces are averages obtained at 0.25 mM, dotted traces at 2 mM $[Ca^{2+}]_0$, more than 300 EPSCs were aligned on the beginning of the stimulation artefact and averaged (top). The time course of the averaged EPSC at low and physiological $[Ca^{2+}]_0$ is similar. In contrast, the average time course of the individual EPSCs at low $[Ca^{2+}]_0$ is clearly faster than that at physiological $[Ca^{2+}]_0$, as seen by aligning EPSCs in low $[Ca^{2+}]_0$ at their 50% rise point (obtained at 1 kHz) before averaging (bottom, 3 kHz).

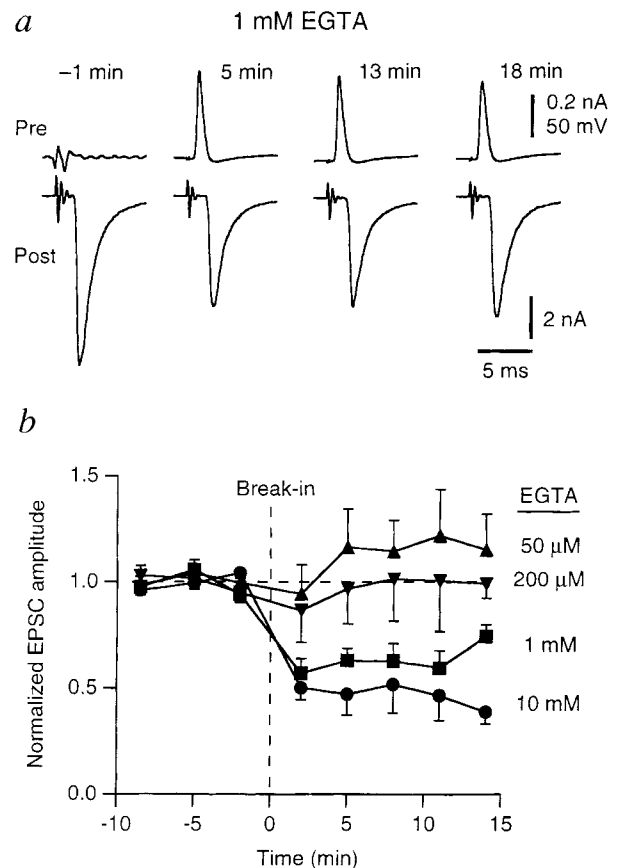


FIG. 4 Dialysis of terminals with EGTA reduces fast synaptic transmission. *a*, Simultaneous pre- and postsynaptic recording in the MNTB. Amplitude of the EPSC (bottom, Post) was reduced after dialysing the terminal with a solution containing 1 mM EGTA, compared to EPSCs elicited in the presence of the endogenous calcium buffer (left, same experiment, 1 min before presynaptic break-in). Times indicated above the traces are relative to the moment of break-in, at which point the presynaptic cell-attached voltage-clamp recording (top left) changed to a whole-cell current-clamp recording (other top traces). *b*, Average effect of different concentrations of EGTA on EPSC amplitudes, averaged over 3 min intervals. EPSCs were normalized to the average amplitude before establishing the whole-cell configuration in the terminal. EPSC amplitudes were stable in most experiments, even after up to 1 h of simultaneous pre- and postsynaptic whole-cell recording. Number of experiments were 9, 3, 4 and 11 for 0.05 (triangles), 0.2 (inverted triangles), 1 (squares) and 10 mM (circles) EGTA, respectively.

Therefore, although the calcium concentration in the immediate vicinity of a single calcium channel may be high enough to induce vesicle fusion, most vesicles are too far away in the MNTB synapse and the high concentrations of calcium thought to be needed to evoke fast transmitter release² can probably only be attained by the simultaneous opening of multiple calcium channels. Additional support is provided by the observation that a high density of calcium channels has been observed at the release face of calyx-type terminals²⁵, and by the supralinear effect of calcium channel blockers on transmitter release in experiments in which the activity of large numbers of terminals in hippocampus or cerebellum were monitored^{6,26,27}.

Synaptic strength may be regulated quite differently when multiple calcium channels contribute to the release of a single vesicle, than when release is dominated by the opening of a single calcium channel. Because of the large number of calcium channels that open, the release of an individual vesicle is less dependent on the stochastic behaviour of individual calcium channels. However, when calcium domains overlap, synaptic strength will be very sensitive to small changes in the concentration and the affinity of mobile presynaptic calcium buffers, as these generally bind Ca^{2+} faster than does EGTA²⁸. □

Methods

Slices were cut from the brainstem of Wistar rats, 8–10 days old, transferred to a recording chamber, and perfused at room temperature (23–24 °C) with a solution containing (in mM): 125 NaCl, 2.5 KCl, 1 MgCl_2 , 2 CaCl_2 , 25 dextrose, 1.25 NaH_2PO_4 , 0.4 ascorbic acid, 3 *myo*-inositol, 2 sodium pyruvate, 25 NaHCO_3 , pH 7.4 when bubbled with 95% O_2 , 5% CO_2 (ref. 5). In low- $[\text{Ca}^{2+}]_0$ experiments, CaCl_2 was replaced by MgCl_2 . Only synapses of which the post-synaptic cells discharged an action potential in response to afferent stimulation were selected for recording⁵. Potentials were corrected for a liquid junction potential of –11 mV between the extracellular and the pipette solution. Holding potential in voltage-clamp experiments was –80 mV. Presynaptic, whole-cell, two-electrode voltage-clamp recordings from the calyces of Held were made with an Axoclamp 2B amplifier, using two HS-2A $\times 0.1\text{L}$ headstages. Pipettes (5–11 M Ω), coated with Sylgard were at least 8 μm apart. Ca^{2+} currents were isolated pharmacologically⁵. As the waveform command we used the presynaptic action potential displayed in Fig. 2a of ref. 5, interpolated to 20 μs per point with a cubic spline. Subtraction of the passive response was by the P/+5 method. The P/+5 current differed by <1% from the P/–5 current and matched the first derivative of the voltage transient well, so it probably represents the sum of the leak current and the current needed to charge the presynaptic membrane. The difference between the P/5 current and the full waveform current was almost completely blocked by Cd^{2+} (0.1 mM, $n = 4$). Experiments were only accepted for analysis if a good voltage clamp could be established (around one-third of total) with 20–80% rise time <100 μs at <10% overshoot. Currents were filtered at 5 kHz and sampled at 50 kHz (ref. 29) with a 16-bit analogue-to-digital converter (Instrutech).

Voltage-clamp recordings from principal cells, presynaptic current-clamp recordings and afferent stimulation were done as described⁵. Series resistance in postsynaptic recordings (<15 M Ω) was always compensated to 98% (lag 10 μs). In presynaptic solutions with different concentrations of EGTA (Roth, Stuttgart), potassium gluconate and potassium glutamate concentrations were adjusted to maintain an osmolarity of $290 \pm 5 \text{ mOsm kg}^{-1}$. With series resistances <30 M Ω , EGTA concentrations in the terminal will be the same as in the pipette within 15 min after break-in⁵.

Afferent stimulation interval was typically 1 min, except at 0.5 and 0.25 mM $[\text{Ca}^{2+}]_0$, where it was 0.2 and 2 Hz, respectively. EPSCs at low $[\text{Ca}^{2+}]_0$ were detected using a pattern detection algorithm³⁰ after digital filtering to 1 kHz. The pattern function used for correlating with the records was

$$p(t) = A[1 + \text{erf}(5.336t)]\exp(-t/0.55),$$

where t is time (ms), A is amplitude, and erf is the error function. It described the quantal EPSCs at 1 kHz well. After subtracting the average of >30 traces without an EPSC, for each point in the trace a linear correlation with the pattern function was done (IGOR, Wavemetrics). Threshold was at 14–27 pA (assuming a perfectly fitting EPSC, with no noise and no offset), depending on baseline noise (s.d. 2.2–5.5 pA). In addition, the Pearson correlation coefficient (r) between the putative EPSCs and the pattern function had to be ≥ 0.8 .

Delay histograms were fitted with a first-order Poisson function, for $t > t_0$:

$$N(t) = A(t - t_0)\exp(-(t - t_0)/\tau)$$

where $N(t)$ is the number of events, A is amplitude, t is time, t_0 is start time, and τ is the time constant.

Quantal content was calculated as the ratio of the average peak amplitude (at 3 kHz) of the EPSCs at 2 mM $[\text{Ca}^{2+}]_0$ and the average peak amplitude (at 1 kHz) of the delayed EPSCs at 0.25 mM $[\text{Ca}^{2+}]_0$. Spontaneous, phasic and delayed EPSCs fell within the 15-ms time interval preceding the stimulus artefact, during the 3 ms following the negative peak of the prespike, and during the following 50 ms, respectively. In addition, the ratio of the charge of the EPSCs at 2 mM $[\text{Ca}^{2+}]_0$ (integrated over 50 ms) and the charge of the delayed EPSCs at 0.25 mM $[\text{Ca}^{2+}]_0$ (20 ms) was taken. Results are given as mean $\pm$ s.e.m.

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CORRESPONDENCE and requests for materials should be addressed to J.G.G.B. (e-mail: borst@sunny.mpiimf-heidelberg.mpg.de).

Superoxide dismutase protects calcineurin from inactivation

Xutong Wang*, Valeria C. Culotta† & Claude B. Klee*

* Laboratory of Biochemistry, National Cancer Institute, NIH, Bethesda, Maryland, 20892-4255, USA

† Division of Toxicological Sciences, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland 21205, USA

CALCINEURIN is the only protein phosphatase known to be under the control of Ca^{2+} and calmodulin^{1,2}. It is targeted by immunosuppressive drugs and has a critical role in T-cell activation^{3,4}. It is specifically inhibited by immunosuppressant immunophilin complexes, which enabled its function in regulating a wide range of cellular responses to Ca^{2+} -mobilizing signals^{5,6} to be identified. Calcineurin *in situ* is 10–20 times more active than in the purified form and is subject to a time- and Ca^{2+} /calmodulin-dependent reversible inactivation that is facilitated by small, heat-stable molecules⁷. Here we identify a factor that prevents the inactivation of calcineurin *in vitro* and *in vivo* as the enzyme superoxide dismutase, which indicates that inactivation may be the result of oxidative damage to the Fe–Zn active centre of calcineurin. The redox state of iron provides a mechanism to regulate calcineurin

activity by desensitizing the enzyme and coupling Ca^{2+} -dependent protein dephosphorylation to the redox state of the cell. The protection of calcineurin against inactivation by superoxide dismutase constitutes a new physiological role for this enzyme which enables the Ca^{2+} -dependent regulation of cellular processes to be modulated by the redox potential.

The bulk of calcineurin activity in a brain extract (calcium-stable calcineurin, or CN_s) becomes stable after removal of small inactivator(s) by gel filtration, but a fraction of calcineurin (calcium-inactivated, or CN_i), which elutes early from the column, remains susceptible to inactivation even after removal of these inactivators (Fig. 1). The CN_i and CN_s fractions each have high specific activities (113 and 146 units mg^{-1} , respectively); however, CN_i loses more than 90% of its phosphatase activity during a 20-min preincubation with Ca^{2+} and calmodulin, whereas CN_s retains more than 60% of its activity after the same treatment. The inactivator(s), eluted at the included volume of the column, have only a small inhibitory effect on the activity of calcineurin samples that have not been preincubated, but completely inactivate CN_s when preincubated with Ca^{2+} . These data indicated that a factor, partially separated from calcineurin by gel filtration, might protect CN_s against inactivation.

To demonstrate the presence of such a factor, CN_s was first inactivated by incubation with partially purified inactivator(s). The inactivated enzyme was applied to a Sephadex G-100 column to remove the inactivator(s) and the column fractions tested for phosphatase activity and their ability to protect CN_i against inactivation (Fig. 2). Only a small peak of phosphatase activity was found in the column eluate, so inactivation of CN_s is not reversed by removal of the inactivator(s). When CN_i was preincubated for 20 min with the same column fractions and Ca^{2+} , a treatment that otherwise almost completely inactivates CN_i , fractions 35–60 protected CN_i from inactivation, revealing the presence of the stabilizing factor.

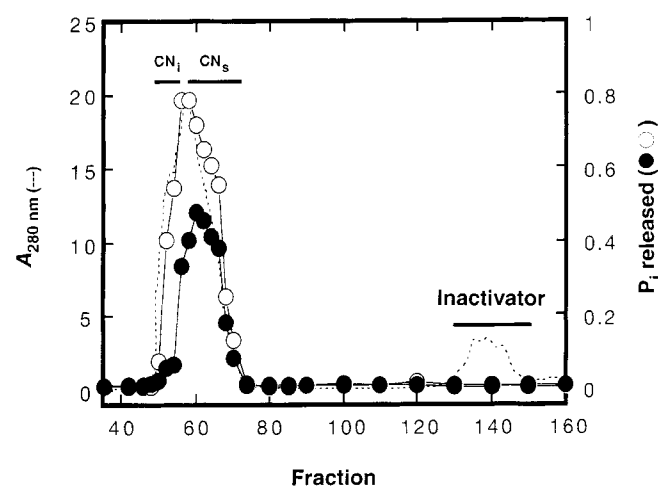


FIG. 1 Stabilization of calcineurin by gel filtration. The 10,000g supernatant of brain homogenate (19.5 ml) was applied to a 2.5×80 cm column of Sephadex G-50 equilibrated with 50 mM Tris-HCl, pH 8, 0.1 mM EDTA and 0.1% mercaptoethanol. Calcineurin activity was measured before (○) and after (●) a 20-min preincubation in the presence of CaCl_2 . Fractions containing Ca^{2+} -stable (CN_s) and Ca^{2+} -inactivated (CN_i) calcineurin were pooled as indicated.

TABLE 1 Ca^{2+} -dependent inactivation of yeast calcineurin

Time (min)	Preincubation Additions	Calcineurin activity	
		WT lysates	SOD1Δ lysates
		(pmol min^{-1} mg^{-1})	
0	Ca^{2+}	25.8 ± 0.2	2.7 ± 0.6
20	Ca^{2+}	2.2 ± 1.2	ND
0	$\text{Ca}^{2+} + \text{SOD}$	29.4 ± 2.4	ND
20	$\text{Ca}^{2+} + \text{SOD}$	26.8 ± 1.1	ND
0	EGTA	27.1 ± 1.4	1.4 ± 1.0
20	EGTA	25.4 ± 0.5	1.4 ± 0.9

Protein phosphatase activity was measured as described in Methods in the presence of 1 mM free Ca^{2+} and 0.1 μM calmodulin, in the presence or absence of 1 μM FK506. If present during preincubation at 30 °C, the concentration of CaCl_2 was $1.5 \text{ mM} \pm 1.6 \mu\text{g}$ per ml SOD and 2 mM for EGTA. FK506-insensitive phosphatase (5–8 and 2–3 pmol per min per mg total protein for the wild-type (WT) and SOD1Δ lysates, respectively) was subtracted from the total phosphatase to determine calcineurin activity. ND, not detectable.

Sufficient quantities of stabilizing factor were purified to allow its identification by peptide mapping and protein sequencing. Reverse-phase high-performance liquid chromatography (HPLC) on a phenyl column yielded a peak of stabilizing activity co-eluting with a single polypeptide of relative molecular mass 18,000 (M_r , 18K) (Fig. 3). After digestion of 18K polypeptide with pepsin at pH 1.6, two resulting peptides, separated by reverse-phase HPLC on a C18 column, were sequenced. Their respective amino-acid sequences were KGDGPVQGV-F and DGVANV-SIEDR, which are identical to sequences found in rat superoxide dismutase^{8,9}, showing that the stabilizing factor must be superoxide dismutase. We were unable to sequence the intact 18K protein by Edman degradation, which indicated that the amino terminus of the stabilizing factor is blocked, as is that of superoxide dismutase. The ultraviolet and visible absorption spectra of the stabilizing factor (Fig. 4) are identical to those of the brain isoform of superoxide dismutase¹⁰. A 680-nm peak in the visible spectrum is a characteristic feature of cytoplasmic Cu-Zn

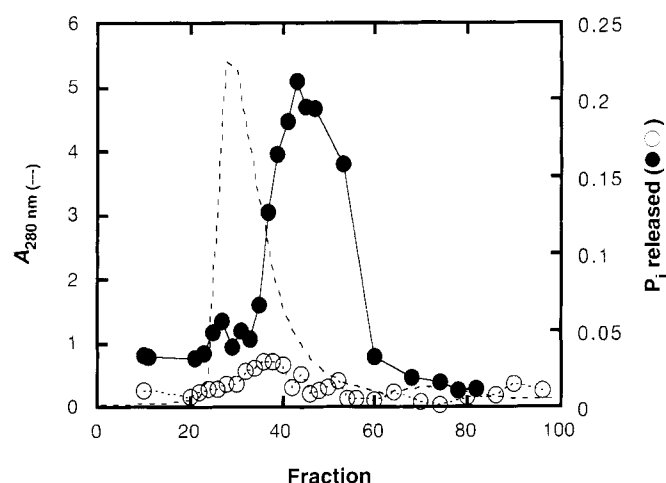


FIG. 2 Detection of a calcineurin-stabilizing factor. CN_s (33 mg protein) was incubated at 30 °C for 30 min in the presence of 1 mM Ca^{2+} , 0.1 μM calmodulin and 0.4 ml 'inactivator' before loading on a 1.5×30 cm Sephadex G-100 column equilibrated with 50 mM Tris-Cl, pH 8.0, 50 mM NaCl, 2 mM DTT and 0.1 mM EDTA. Calcineurin activity was measured without prior incubation (○); column fractions, tested for their ability to protect CN_i against inactivation, were incubated in the presence of 1 mM Ca^{2+} for 20 min with CN_i (0.012 units) before addition of the substrate to measure CN_i activity (●).

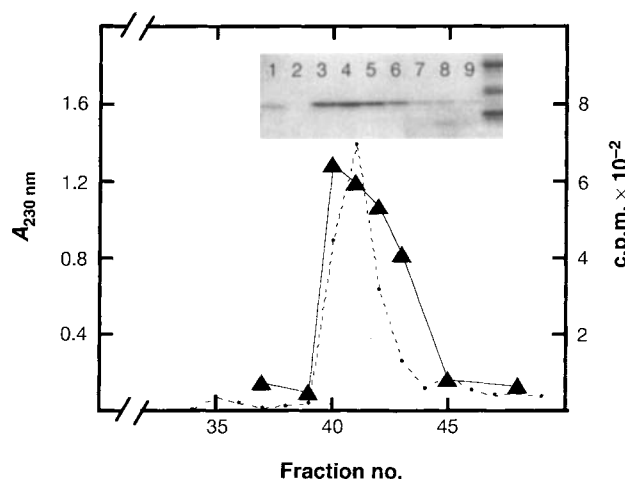


FIG. 3 Purification of stabilizing factor by HPLC. After gel filtration, the stabilizing factor was purified by HPLC as described in Methods. Absorbance was monitored at 230 nm (dotted line) and the stabilizing activity (c.p.m.) determined as described in Fig. 2 legend. Fractions 38–45 were subjected to SDS-PAGE and the Coomassie-blue-stained patterns are shown in lanes 1–9. M_r markers are: lane 1, calcineurin B; M_r 18K; right lane, carbonic anhydrase (top band; 30.5K), soybean inhibitor (21K) and lysozyme (bottom; 14.4K).

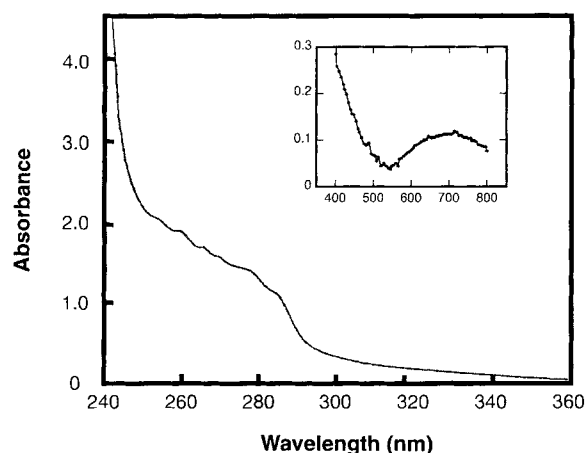


FIG. 4 Ultraviolet and visible absorption spectra of the stabilizing factor. The concentrations of the protein solutions in 40 mM Tris-Cl, pH 8.0, containing 0.1 M NaCl, 0.1 mM DTT and 0.01 mM EDTA were 0.7 and 5.6 mg ml⁻¹ for the ultraviolet and visible regions, respectively.

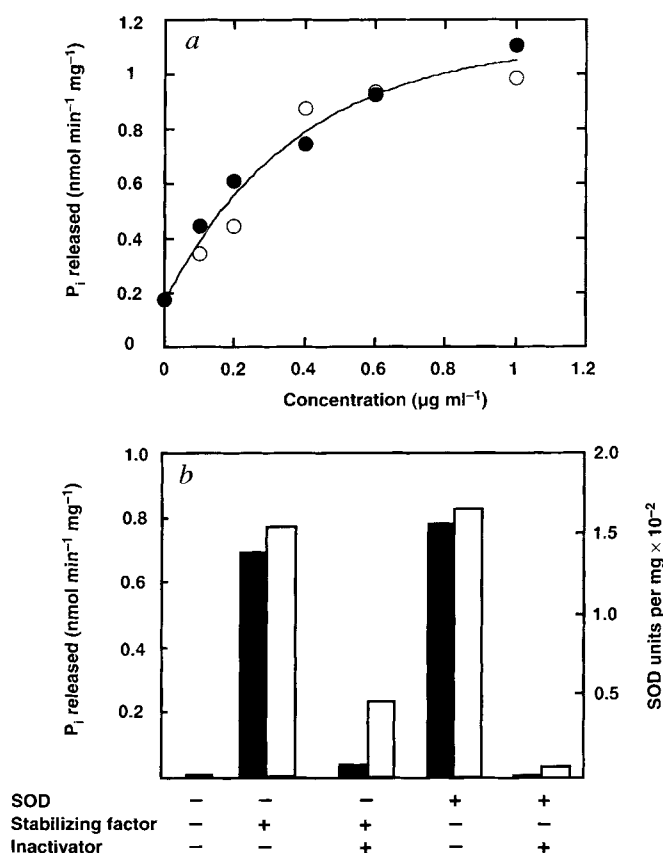


FIG. 5 Comparison of activities of stabilizing factor and superoxide dismutase. *a*, CN_i was preincubated for 20 min in the presence of the indicated concentrations of superoxide dismutase (●; Sigma) or HPLC-purified stabilizing factor (○) and assayed for calcineurin activity. *b*, Protein phosphatase activity of CN_i (black bars) was measured after 20 min preincubation in the presence of Ca²⁺/calmodulin with or without added factors. The superoxide dismutase activities of the stabilizing factor and rat erythrocyte dismutase (white bars) were measured in the presence or absence of 40 μl per ml inactivator.

superoxide dismutases⁹. Like superoxide dismutase, the native factor is a dimer with a relative molecular mass, determined by sedimentation equilibrium, of 31,000 ± 1,500.

Commercially available purified erythrocyte superoxide dismutase was as potent as the stabilizing factor in protecting CN_i against inactivation (Fig. 5*a*). The similar superoxide dismutase-specific activities of the stabilizing factor and of rat erythrocyte superoxide dismutase confirmed the identity of the stabilizing factor (Fig. 5*b*). The protection by superoxide dismutase was prevented in the presence of the low- M_r inactivator(s), and the loss of the protective effect was correlated with inhibition of the superoxide dismutase activity (Fig. 5*b*). Thus, the small inactivator(s) act by inhibiting superoxide dismutase rather than by acting directly on calcineurin. We are currently investigating the identity of the low- M_r inactivators and the mechanism of inhibition.

The protection of calcineurin by superoxide dismutase resembles that of the [4Fe-4S] cluster enzymes that are damaged by superoxide anions^{11,12}. As expected, calcineurin is inactivated by paraquat, a compound that generates superoxide anions (data not shown). We believe that superoxide dismutase probably protects calcineurin by preventing oxidation of the iron at its catalytic centre. There is good evidence that calcineurin is an iron-zinc-containing enzyme: for example, purified calcineurin contains substoichiometric amounts of iron and zinc^{13–15} and these two metal ions are included in crystal structures of calcineurin^{16,17} modelled on the structure of kidney bean [Fe(III)-Zn(II)] purple acid phosphatase¹⁸. The [Fe(III)-Zn(II)] centre of this latter enzyme acts as a Lewis acid and is critical for catalysis. A similar mechanism may operate in calcineurin-catalysed dephosphorylation^{19,20}. The oxidation state of iron in this reaction is inconclusive: one study indicates that Fe(II)-calcineurin is the active form of the enzyme¹⁴, whereas similar (but low) specific activities for the Fe(II) and Fe(III) forms of the enzyme have also been reported^{15,16}. Our results are more consistent with the first possibility: crude calcineurin was protected against inactivation by ascorbate and was found to retain 45% of its activity when preincubated in an anaerobic chamber for 30 min at 25 °C, whereas the same treatment under aerobic conditions resulted in a complete loss of activity. Reactivation required the addition of 0.5 mM ferrous ammonium sulphate in the presence of 5 mM dithiothreitol (DTT) and 5 mM sodium ascorbate (Fig. 6).

Accessibility of the catalytic centre of calcineurin may explain

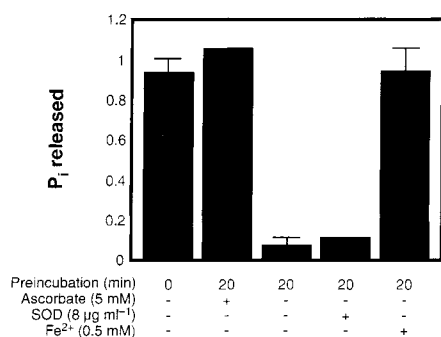


FIG. 6 Protection of calcineurin by ascorbate and reactivation by Fe²⁺. CN_i was assayed for calcineurin activity (P_i released per min per mg) before and after a 20-min preincubation in the absence or presence of 5 mM ascorbate as indicated, and the reaction was started by addition of substrate. When present, superoxide dismutase (8 µg ml⁻¹) or ferrous ammonium sulphate (0.5 mM) was added just before substrate.

its dependence on Ca²⁺ and calmodulin for inactivation. The three-dimensional structure of calcineurin indicates that its auto-inhibitory domain masks the metal ions at the active site¹⁷. The activation of calcineurin by Ca²⁺/calmodulin probably results from the displacement of this autoinhibitory domain¹, so exposure of the catalytic centre upon binding of Ca²⁺/calmodulin renders it open to oxidative attack by the superoxide anion.

To investigate the protective effect of superoxide dismutase *in vivo*, we asked whether two independent signalling pathways affected by deletion of calcineurin in yeast, the recovery from G1 arrest after pheromone treatment²¹ and the adaptation to a high-salt stress^{22,23}, are protected by superoxide dismutase in intact yeast cells. When grown under aerobic but not anaerobic conditions, yeast *SOD1*-null mutants, which lack Cu-Zn superoxide dismutase, were unable either to recover from G1 arrest induced by α -factor, or to grow in the presence of 1 M NaCl or 0.2 M LiCl (results not shown). Inactivation of calcineurin, at least in part, explains these *in vivo* effects of *SOD1* deletion. The activity of calcineurin in lysate of *SOD1* mutants grown under aerobic conditions is only one tenth of that of wild-type cells. In the latter case, as in calcineurin from crude brain extracts, calcineurin is subject to a Ca²⁺-dependent inactivation that is prevented by superoxide dismutase (Table 1).

The Ca²⁺-dependent oxidation of a redox metal that participates in the mechanism of action of calcineurin provides a reversible mechanism for the desensitization of calcineurin after prolonged stimulation with Ca²⁺ and a way to couple Ca²⁺ signalling to the oxidation state of the cell. Thus, the activity of calcineurin is regulated by both Ca²⁺-mediated external signals and the intracellular redox potential. The protective effect of superoxide dismutase against inactivation of protein dephosphorylation reveals a new role for this enzyme in signal transduction phosphorylation cascades. Such effects could be associated with diseases caused by superoxide dismutase mutations such as, for example, familial amyotrophic lateral sclerosis²⁴. □

Methods

Tissue and cell extracts. Rat brain homogenates were freshly prepared as described⁷. Isogenic strains of *Saccharomyces cerevisiae* WT1783 (*MATa leu2-1 112 ura3-1 trp1 his4, kanamycin*) and KS105 (*MATa sod1Δ::LEU2 ura3-1 trp1 his4 kanamycin*) were grown for 24 h at 30 °C in YEPD medium under aerobic conditions²⁵. Cells were collected and washed with 0.1 M Tris-HCl (pH 8.0), 10 mM EDTA and 2 mM DTT, suspended in 0.1 M Tris-HCl (pH 7.5) containing 2 mM EDTA, 2 mM EGTA, 2 mM DTT and protease inhibitors (per ml: 50 µg soybean inhibitor, 10 µg aprotinin, 10 µg leupeptin, 50 µg PMSF and the lysates prepared as in ref. 21. Protein and calcineurin concentrations were determined as described⁷.

Enzymatic assays. Protein phosphatase activity was measured according to ref. 26 in the presence of okadaic acid to inhibit protein phosphatases 2A and without MgCl₂ to prevent activation of protein phosphatase 2C (ref. 27). Enzyme aliquots in 40 µl of buffer A (40 mM Tris-Cl, pH 8.0, for brain calcineurin or 40 mM MES-NaOH, pH 6.5, for yeast calcineurin, 0.1 M KCl, 0.5 µM okadaic acid and 0.4 mg ml⁻¹ bovine serum albumin) containing 1 mM DTT and 0.1 µM calmodulin were immediately mixed with 20 µl of a 3 µM solution of phospho-

peptide²⁸ dissolved in buffer A containing 2 mM CaCl₂ or incubated for 20 min at 30 °C in the presence of 1 mM CaCl₂ before addition of the substrate. Release of phosphate was measured at 30 °C for 2 min. One unit of enzyme catalyses the release of 1 nmol P_i min⁻¹ under these conditions. Inhibition by the calmodulin-binding peptide M13 (ref. 29) and by FK506 (ref. 5) was used to demonstrate that the phosphatase was calcineurin. Superoxide dismutase was assayed by inhibition of the reduction of nitroblue tetrazolium by xanthine oxidase³⁰. Incubation mixtures contained 40 ng ml⁻¹ superoxide dismutase or stabilizing factor in 50 mM Na₂CO₃, pH 10.2, 0.1 mM EDTA, 0.1 mM xanthine and 0.025 mM nitroblue tetrazolium; the reaction was started by addition of 2.8 µg ml⁻¹ xanthine oxidase.

Purification of the stabilizing factor. The supernatant fluid from the homogenate of 25 frozen brains from Sprague Dawley rats in 50 mM Tris-Cl, pH 8.0, 50 mM NaCl, 0.1 mM EDTA and 0.5 mM DTT, was passed through a 2.5 × 50 cm DE-23 cellulose column to remove calcineurin and calmodulin. The stabilizing factor, recovered in the column flow-through, was concentrated by vacuum dialysis to remove the small inactivators and the pH adjusted to 5.2 by dialysis against 50 mM sodium acetate buffer containing 50 mM NaCl, 0.1 mM EDTA and 0.5 mM DTT. The stabilizing factor applied to a 2.5 × 10 cm CM-52 cellulose column equilibrated in the same buffer was recovered in the column flow-through concentrated and gel-filtered (Fig. 2 legend). Fractions containing the stabilizing factor were pooled and applied to 3.9 × 300 mm Bondapak (10-µm resin diameter) phenyl column (Waters Associates) and the column eluted with a 0–50% linear gradient of acetonitrile in 0.01 M potassium phosphate, pH 6.0, over 30 min. The flow rate was 1 ml per min and 0.5-ml fractions were collected.

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CORRESPONDENCE and requests for materials should be addressed to C.B.K. (e-mail: chl@heix.nih.gov).

Ligand binding regulates the directed movement of $\beta 1$ integrins on fibroblasts

Dan P. Felsenfeld, Daniel Choquet & Michael P. Sheetz

Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710, USA

To enable cells to crawl, adhesion receptors such as integrins must bind to extracellular molecules and simultaneously interact with force-generating components of the cytoskeleton^{1,2}. We show here that the binding of extracellular ligand in living cells induces the attachment of $\beta 1$ integrins to the retrograde-moving cytoskeleton. Unliganded integrins are not associated with the rearward-moving cytoskeleton: gold particles attached to $\beta 1$ integrin by a monoclonal antibody diffuse in the membrane. However, addition of soluble RGD peptide (single-letter amino-acid code) or the use of fibronectin-coated gold particles causes the attachment of integrins to the rearward-moving cytoskeleton. Deletion of the $\beta 1$ cytoplasmic tail blocks cytoskeletal attachment. The directed movement of integrins in response to ligand indicates that ligand binding is the critical step in regulating organized receptor movement on the cell surface and the migration of adherent cells.

Integrins are a family of $\alpha\beta$ heterodimeric transmembrane proteins that mediate cell adhesion in a variety of motile cell types^{3,4}. In translocating cells, the directed movement of integrin populations in the membrane has been observed using antibodies coupled to fluorescent dyes⁵, indicating that the organized movement of integrins on the cell surface may play a role in mediating cell translocation. However, little is known about the regulation of integrin association with dynamic components of the cytoskeleton, a necessary step for directed receptor movement.

Small gold particles (40 nm) attached to $\beta 1$ integrins by a non-perturbing antibody, ES66-8 (refs 6, 7), diffuse randomly on lamellipodia of 3T3 cells, with occasional excursions to the leading edge (Fig. 1a, c, e and g; ref. 7). The mean-square displacement (MSD) of 40-nm particles is almost linear with respect to time (Fig. 1g), consistent with random diffusion in the absence of directed movement. In contrast, 1- μ m latex particles coated with high concentrations of the same antibody bind to the cell surface and move centripetally, away from the leading edge (ref. 7, and D.C., D.P.F. and M.P.S., manuscript in preparation). However, at low concentrations of ES66-8, even 1- μ m beads diffuse on the cell surface. Together these results suggest that crosslinking can direct the association of a receptor with the underlying cytoskeleton, although with insufficient crosslinking, $\beta 1$ integrins diffuse freely on the cell surface.

Proteins of the extracellular matrix are known to regulate interactions between their integrin receptor and the cytoskeleton^{6,8-11}, although the dynamics and organized movement of integrins in response to ligand activation have not been characterized. To examine whether fibronectin binding could directly modulate the interaction between integrins and dynamic components of the cytoskeleton, 40-nm gold particles were coated with the integrin-binding domain of fibronectin (fibronectin type III domains 7-10; FNIII7-10; refs 12, 13). Unlike antibody-coated gold particles, FNIII7-10-coated particles moved centripetally on the cell surface at a rate similar to that of larger antibody-coated 1- μ m beads (3-10 μ m min⁻¹, Fig. 1b, d, f and h). After binding, FNIII7-10-coated particles diffused briefly (<1 s) before beginning directed movement (Fig. 1d). The degree of diffusion then continued to decrease as the bead moved away from the leading edge of the cell, indicating that the strength of integrin-cytoskeleton linkage, or the number of integrins bound to ligand on the bead, can be dynamically regulated by ligand binding. In this respect, FNIII7-10-coated gold particles differ from large

antibody-coated beads, which do not show decreased diffusion over time (D.C., unpublished observations).

Interactions between integrins and the cytoskeleton are thought to be mediated by the cytoplasmic domain of the integrin $\beta 1$ subunit^{4,14}. To determine whether structures in the cytoplasmic domain of the $\beta 1$ subunit have a role in ligand-activated receptor movement, we examined 3T3 cells transfected with either a truncated (765T; cytoplasmic domain deleted from the first intracellular residue) or full-length ($\beta 1$ C) form of the chick $\beta 1$ -integrin subunit^{7,8,15,16}. These cells also express an endogenous mouse $\beta 1$ -subunit to which ligand-coated beads can potentially bind¹⁵. FNIII7-10-coated gold particles spend longer diffusing on the surface of 765T cells than on $\beta 1$ C cells, before beginning directed, retrograde movement (765T, 3.63 ± 2.08 s; $\beta 1$ C, 0.511 ± 0.39 s; all values given as mean $\pm$ s.d.; Fig. 2a-c). This observation is consistent with a role for the cytoplasmic domain of the $\beta 1$ subunit in mediating ligand-activated receptor movement. However, ligand-coated particles on 765T cells eventually

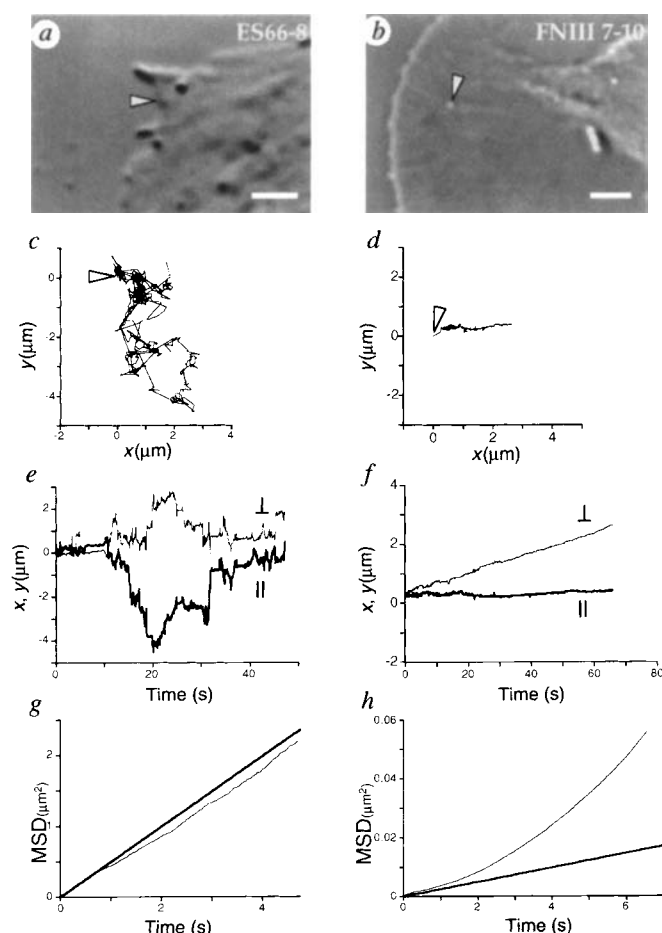
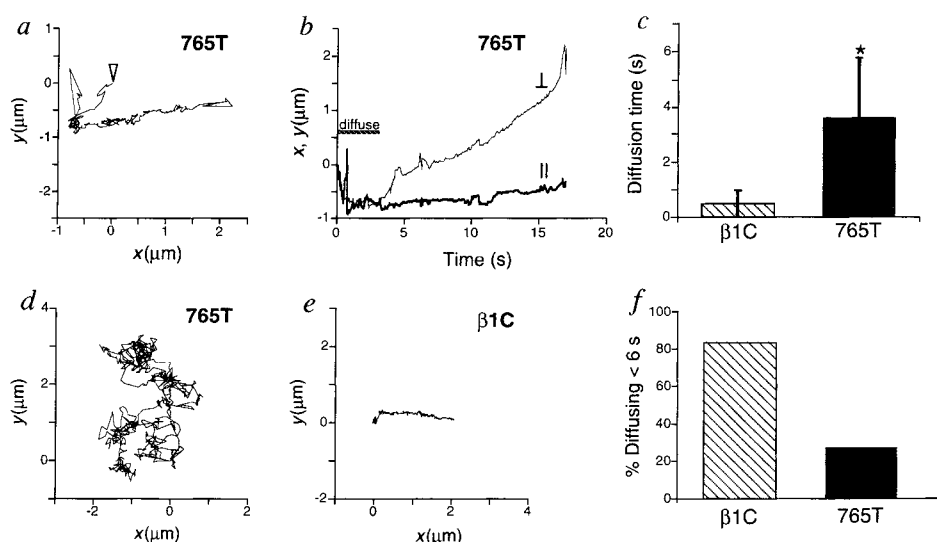


FIG. 1 Fibronectin binding regulates the association of cell-surface integrins with retrograde-moving cytoskeleton. 40-nm colloidal gold particles coated with either ES66-8, an antibody against chick $\beta 1$ integrin subunit⁶ (a, c, e, g), or FNIII7-10 (b, d, f, h), bound to the surface of 3T3 fibroblasts transfected with a cDNA encoding the chick $\beta 1$ integrin subunit. Antibody-coated beads diffuse in the plane of the membrane whereas FNIII7-10-coated beads move with little diffusion, away from the leading edge. a, b, Video micrographs showing bead position (arrowhead) just after bead binding (at time zero; scale bar, 2.5 μ m). c, d, Bead trajectory (x versus y; bead position at time zero indicated by arrowhead). The y-axis is roughly parallel to the edge of the lamellipodia. e, f, x (fine line; $\perp$) and y (bold line; $\parallel$) coordinates with respect to time. g, h, Mean-square displacement (MSD; fine line) with respect to time. Bold line shows predicted curve for a purely diffusing particle moving with the same diffusion coefficient.

FIG. 2 Deletion of the integrin $\beta 1$ subunit cytoplasmic domain interferes with ligand-activated receptor movement. FNIII7-10-coated gold bound to 3T3 cells expressing a truncated chick $\beta 1$ subunit lacking all cytoplasmic residues (765T) or a full-length chick $\beta 1$ subunit ($\beta 1C$) in the absence (a-c) or presence (d-f) of inhibitory antibodies^{8,16,17}. **a**, Bead trajectory (x vs y; bead position at time zero indicated by arrowhead) of FNIII7-10-gold on 765T cells. **b**, Coordinates x (fine line, $\perp$) and y (bold line; $\parallel$) plotted with respect to time. FNIII7-10-coated particles spend longer diffusing on 765T cells (hatched line, first 4 s) compared with $\beta 1C$ cells before beginning directed movement. **c**, Average measurement of initial time spent diffusing by FNIII7-10 particles on $\beta 1C$ cells ($n = 3$) and 765T cells ($n = 12$). Data are mean $\pm$ s.d.; $P < 0.0006$. **d**, **e**, Bead trajectory (x vs y) of FNIII7-10-gold on the surface of 765T (**d**) or $\beta 1C$ (**e**) cells in the presence of an inhibitory polyclonal antiserum directed against rodent $\beta 1$ integrins (Lenny). In most cases, beads on 765T diffused for most of the period of observation (~ 60 s) whereas beads on $\beta 1C$ began retrograde movement with little or no delay. **f**, Percentage of FNIII7-10 gold that diffused for less



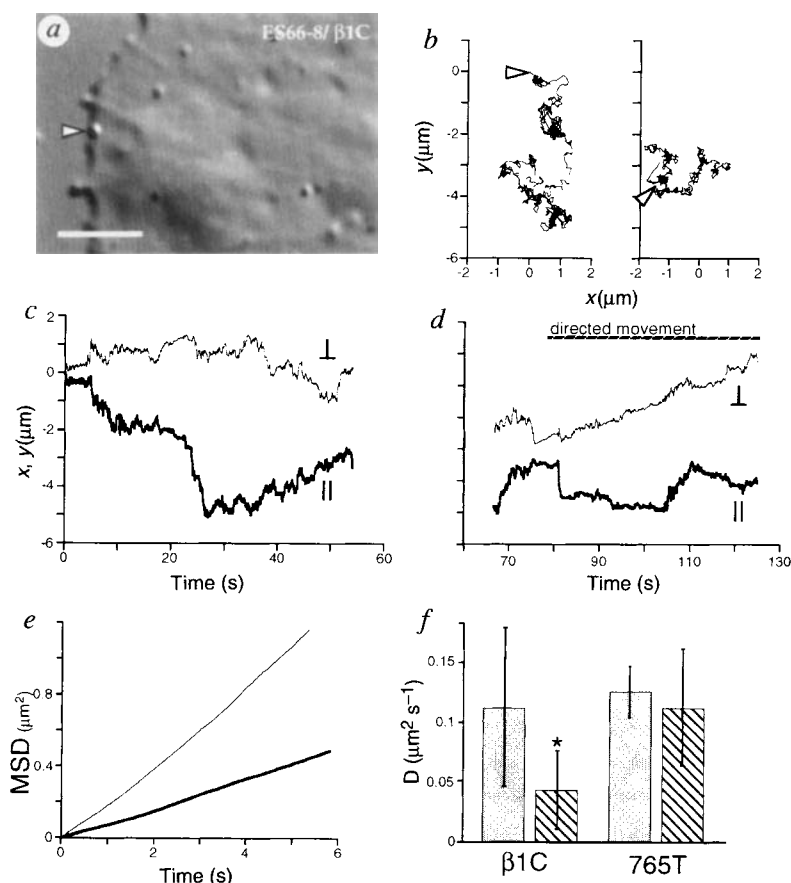
than 6 s before beginning retrograde movement. Experiments were performed in the presence of inhibitory antiserum on 765T ($n = 11$) and $\beta 1C$ cells ($n = 12$).

exhibited directed movement, indicating either that several integrins are binding to the same particle or that the truncated receptor may be able to mediate some interactions with the cytoskeleton.

To distinguish between these two possibilities, we examined the behaviour of 765T cells cultured in the presence of a polyclonal antibody (Lenny¹⁷), which selectively recognizes the mouse $\beta 1$

subunit and inhibits ligand binding. To remove antibodies that crossreact with the transfected chick receptor, diluted antiserum was preadsorbed against chick embryo fibroblast (CEF) cells. Preadsorbed antiserum caused cell rounding of 765T cells cultured on fibronectin, but had no significant effect on either $\beta 1C$ cells or CEF cells (data not shown), indicating that this antiserum

FIG. 3 Soluble ligand causes the reversible attachment of $\beta 1$ integrins to rearward-moving cytoskeleton. Gold particles attached to chick $\beta 1$ integrins on $\beta 1C$ cells by ES66-8 antibody (**b** (left), **c**, **e**, **f**) show a significantly decreased diffusion-coefficient on addition of RGD-containing peptides (**b** (right), **d**, **e**, **f**; GRGDS $200 \mu\text{g ml}^{-1}$ or GRGDTP $100 \mu\text{g ml}^{-1}$; ref. 18). **a**, Video micrographs showing bead position (arrowhead) at time zero (scale bar, $5 \mu\text{m}$). **b**, Trajectory for a single particle before (left) and after (right) peptide (bead position at time zero indicated by arrowhead). **c**, **d**, x (fine line, $\perp$) and y (bold line; $\parallel$) coordinates with respect to time before (**c**) and after peptide (**d**, peptide added at 60 s). Retrograde movement apparent in x position after peptide addition (**d**, hatched bar; 75 s to end). **e**, Decrease in diffusion coefficient after the addition of peptide is apparent from decrease in slope of MSD plot (fine line, no peptide; bold line, peptide). **f**, Comparison of diffusion coefficient (mean $\pm$ s.d.) of antibody-coated gold particles on $\beta 1C$ cells (no peptide; $D = 0.11 \pm 0.066 \mu\text{m}^2 \text{s}^{-1}$; $n = 10$; peptide; $D = 0.043 \pm 0.032 \mu\text{m}^2 \text{s}^{-1}$, $n = 6$) and 765T cells (no peptide; $D = 0.12 \pm 0.021 \mu\text{m}^2 \text{s}^{-1}$, $n = 4$; peptide; $D = 0.11 \pm 0.048 \mu\text{m}^2 \text{s}^{-1}$, $n = 4$). Grey bars, no peptide; hatched bars, peptide. Diffusion coefficient shows a significant (2-fold) reduction on $\beta 1C$ ($P < 0.03$) but no change on 765T cells.



selectively inhibits the mouse $\beta 1$ subunit in these cells. FNIII7–10-coated gold particles bound to the surface of 765T cells in the presence of inhibitory antiserum and diffused freely, often for the entire period of observation >60 s (Fig. 2d and f). Under the same conditions, ligand-coated particles began retrograde movement with almost no delay (Fig. 2e, f). This result strongly suggests that the ligand-induced directed movement of integrins is mediated by the cytoplasmic domain of the $\beta 1$ subunit.

Although the ligand FNIII7–10 on 40-nm gold particles regulates the directed movement of integrins, it is possible that the greater diffusive mobility of antibody-coated beads reflects a difference in the crosslinking ability of these particles. Therefore, we added soluble integrin ligands, the peptides GRGDS ($200 \mu\text{g ml}^{-1}$) or GRGDTP ($100 \mu\text{g ml}^{-1}$), to beads coated with the antibody ES66-8, which recognizes only the exogenous chick $\beta 1$ -subunit on $\beta 1$ C cells^{6,7,12,18}. We found that particles alternated between higher and lower rates of diffusion, consistent with an abrupt shift from free to immobilized receptor. The diffusion coefficient (D) of antibody-coated gold particles (Fig. 3a, b (left), c, e, f; $D = 0.11 \pm 0.066 \mu\text{m}^2 \text{s}^{-1}$) decreased significantly upon addition of peptide (Fig. 3b (right), d–f; $D = 0.043 \pm 0.032 \mu\text{m}^2 \text{s}^{-1}$; $P < 0.03$), reflecting an overall increase in the percentage of time that a particle spent at the lower diffusion rate. Moreover, bead movement became aligned perpendicular to the leading edge of the cell (Fig. 3d; 75 s to end). In contrast, soluble ligand had no effect on the movement of antibody-coated particles on 765T cells (Fig. 3f; $D = 0.12 \pm 0.021 \mu\text{m}^2 \text{s}^{-1}$ without peptide; $0.11 \pm 0.048 \mu\text{m}^2 \text{s}^{-1}$ with peptide). These results indicate that occupancy of the ligand-binding site on integrins can regulate the directed movement of integrins on the cell surface. As 765T could not be activated by soluble ligand, it is likely that the cytoplasmic domain of $\beta 1$ is involved in mediating ligand-activated receptor movement, although the failure to activate 765T may reflect a reduced affinity of the truncated receptor for ligand. Nonetheless, taken together with the behaviour of ligand-coated beads on 765T cells, this result implicates the cytoplasmic domain of $\beta 1$ in the mediation of ligand-activated integrin movement. As soluble ligand can promote the directed movement of antibody-coated beads on $\beta 1$ C cells, crosslinking ability alone does not explain the different behaviour of FNIII7–10- and antibody-coated particles; however, a combination of crosslinking and ligand activation may be needed to induce directed integrin movement.

The behaviour of FNIII7–10-coated beads on the surface of 765T cells indicates that ligand-coated particles probably interact with more than one receptor simultaneously. FNIII7–10-coated beads on the surface of 765T cells attach to the cytoskeleton more slowly than on $\beta 1$ C cells. However, all of these particles eventually show directed movement, which must be mediated by full-length receptor. Moreover, particles almost never dissociated from the cell surface under these conditions over the course of an experiment (>60 s), suggesting that a single bead is not just hopping

from one receptor to the next. Together, these observations indicate that particles bind to several integrins simultaneously.

The regulation of adhesion-receptor movement on the cell surface is critical to cell translocation. In their default state, integrins diffuse freely on the cell surface^{6,7}, so adhesion receptors at the leading edge of the cell need to engage the force-generating machinery of the cytoskeleton when an appropriate molecule is encountered on the substrate. Our results suggest that the binding of ligands such as fibronectin to their integrin receptors can directly instruct the receptor to attach to the cytoskeleton and begin retrograde movement. When receptor and ligand are anchored to the substratum, ligand binding gives rise to productive force generation and forward cell movement. The ability of cells to regulate receptor–cytoskeletal interactions at the level of individual or small numbers of receptors also has important implications for guided cell movement; by responding focally to a spatially restricted substrate molecule, a cell may be able to reorientate its direction of migration. $\square$

Methods

Bead preparation. 40-nm colloidal gold particles (EY labs) were coated with ovalbumin to neutralize surface charge. Coated particles were then reacted with sulphasuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-biotin; Pierce), and bound with avidin neutralite (Molecular Probes) to permit the binding of biotinylated, recombinant FNIII7–10 or antibody. Antibody (ES66-8; ref. 6) was also directly coupled to gold particles⁷.

Video experiments. NIH3T3 cells transfected with complementary DNA encoding the chick integrin $\beta 1$ subunit^{8,16} were cultured on silanized, laminin-coated coverslips in phenol-red-free DMEM containing 10% calf serum⁷. Prepared gold was added to culture medium at the beginning of the experiment and cells were monitored by video differential-interference contrast microscopy to follow freshly bound particles. ES66-8-coated particles show little or no binding to untransfected 3T3 cells (data not shown). Control gold particles prepared without FNIII7–10 or antibody show little or no binding to the cell surface (data not shown). Particle movement was tracked by computer, using nanometer-resolution techniques^{19–22}.

Mean-square-displacement calculations. Mean-square displacement (MSD) with respect to time was calculated for each particle trace, and two-dimensional diffusion coefficients were calculated by fitting MSD curves with the equation $\text{MSD} = 4Dt + v^2t^2$, or by fitting the first 0.5 s of the MSD curve with a line^{20–22}.

Antibody-inhibition experiments. Antiserum 'Lenny', an inhibitory rabbit polyclonal raised against purified rodent $\beta 1$ integrin¹⁷, was diluted in medium (1:100) and preadsorbed against chick embryo fibroblasts for 30 min at 37 °C to remove crossreactive antibodies to chick antigens. The diluted antiserum was centrifuged to remove cells and debris and added to video cultures just before the addition of FNIII7–10 gold. 3T3 cell lines were cultured on human vitronectin (Collaborative Biomedical) for these experiments (Fig. 2d–f) only. P values were calculated using a two-tailed Student's t -test.

Peptide-ligand experiments. Soluble peptide was run into the chamber after a number of antibody-coated particles had bound to the cell surface. Diffusion coefficients in the presence of peptide were calculated based on particles that had been bound to the cell surface for less than 1 min or on freshly bound particles.

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CORRESPONDENCE and requests for materials should be addressed to M.P.S. (e-mail: Mike_Sheetz@cellbio.duke.edu).

The serpin PAI-1 inhibits cell migration by blocking integrin $\alpha_v\beta_3$ binding to vitronectin

Steingrímur Stefánsson & Daniel A. Lawrence

Biochemistry Department, J. H. Holland Laboratory, American Red Cross, 15601 Crabbs Branch Way, Rockville, Maryland 20855, USA

DURING wound healing, migrating cells increase expression of both the vitronectin receptor (VNR) integrins¹ and plasminogen activators^{2,3}. Here we report that vitronectin significantly enhances the migration of smooth muscle cells (SMCs), and that the specific VNR $\alpha_v\beta_3$ is required for cell motility. We also show that the $\alpha_v\beta_3$ attachment site on vitronectin overlaps with the binding site for plasminogen activator inhibitor (PAI)-1, and that the active conformation of PAI-1 blocks SMC migration. This effect requires high-affinity binding to vitronectin, and is not dependent on the ability of PAI-1 to inhibit plasminogen activators. Formation of a complex between PAI-1 and plasminogen activators results in loss of PAI-1 affinity for vitronectin and restores cell migration. These data demonstrate a direct link between plasminogen activators and integrin-mediated cell migration, and show that PAI-1 can control cell-matrix interactions by regulating the accessibility of specific cell-attachment sites. This indicates that the localization of plasminogen activators at sites of focal contact does not initiate a proteolytic cascade leading to generalized matrix destruction, but instead is required to expose cryptic cell-attachment sites necessary for SMC migration.

The interaction between cells and their substrata is an important regulator of cellular function⁴⁻⁶. During wound healing, migrating cells exhibit enhanced expression of VNR integrins^{1,7,8}, including $\alpha_v\beta_3$, which is transiently expressed at the leading edge of cells invading a fibrin clot¹. Urokinase plasminogen activator (UPA) is located at the leading edge of migrating keratinocytes during the early stages of reepithelialization³, and migrating vascular cells show elevated expression of UPA and its receptor (UPAR), which localize to focal contacts^{2,9}. Vitronectin enhances this colocalization¹⁰, and also accelerates the association of the VNR with vinculin at focal contacts¹¹. Thus, during wound healing, cells show similar expression of UPA and $\alpha_v\beta_3$, both temporally and spatially, suggesting that they may be linked. *In vivo*, UPA and its inhibitor PAI-1 are known to regulate vascular wound healing¹². Mice deficient in UPA are protected from the formation, after vascular injury, of neointima, the area of accumulated cells and associated matrix between the endothelium and the internal elastic lamina of a blood vessel. However, PAI-1 null mice

exhibit excessive intimal thickening owing to SMC migration and proliferation, and overexpression of PAI-1 reduces neointima formation to levels similar to those of UPA null mice. This has long been thought to mean that PAs are required to initiate a proteolytic cascade at the cell-substratum interface that results in matrix destruction, which is essential for cellular migration and invasion¹³. However, here we present data suggesting a more subtle role for plasminogen activators during wound healing, and demonstrate a direct link between plasminogen activators and the VNR integrins.

The PAI-1 binding site has been localized to the first 50 residues of vitronectin¹⁴, a region that also contains the Arg Gly Asp (RGD) cell-attachment site. To determine whether these binding sites overlap, competition studies between purified, radiolabelled VNR and PAI-1 for binding to vitronectin were performed. Wild-type PAI-1 competed efficiently with ¹²⁵I-VNR for binding to immobilized vitronectin (Fig. 1). A mutant PAI-1 (R346A) that binds to vitronectin normally, but does not inhibit plasminogen activators, inhibited the binding of ¹²⁵I-VNR to vitronectin in a manner identical to that of wild-type PAI-1 ($K_i \sim 4$ nM). However, a second PAI-1 mutant (Q123K) that inhibits plasminogen activators normally, but has a significantly reduced affinity for vitronectin¹⁵, was a relatively poor inhibitor of ¹²⁵I-VNR binding to vitronectin. Thus PAI-1 binding to vitronectin is sufficient to block VNR binding. None of the PAI-1s had any effect on the binding of ¹²⁵I-VNR to fibronectin, indicating that the interaction is specific for vitronectin, and that the loss of VNR binding is not due to interactions between PAI-1 and the VNR (data not shown).

PAI-1 undergoes profound conformational changes on reaction with a proteinase^{16,17}, resulting in loss of high affinity for vitronectin¹⁸ and rapid clearance of the PAI-1:proteinase complex by members of the low-density lipoprotein (LDL) receptor family¹⁹. Therefore, to examine the possibility that plasminogen activators might regulate integrin attachment by decreasing the affinity of PAI-1 for vitronectin, competition assays were performed to determine whether the presence of UPA had any effect. In the presence of a twofold molar excess of UPA, the ability of wild-type PAI-1 to inhibit ¹²⁵I-VNR binding to vitronectin was largely abrogated (Fig. 2). These data are consistent with the PAI-1:UPA complex having a significantly reduced affinity for vitronectin, allowing the VNR receptor to displace the inactive complex. In contrast, UPA did not reduce the inhibition of ¹²⁵I-VNR binding to vitronectin by the mutant R346A PAI-1. Thus UPA enhances VNR binding by forming a complex with wild-type PAI-1, and not through proteolysis of either the VNR or vitronectin.

FIG. 1 PAI-1 inhibition of ¹²⁵I-VNR binding to vitronectin. Plot of ¹²⁵I-VNR bound to vitronectin against the concentration of PAI-1 added, as follows: wild-type PAI-1 (filled circles), R346A PAI-1 (filled triangles) and Q123K PAI-1 (open circles). Data represent the average of 5 experiments performed in duplicate.

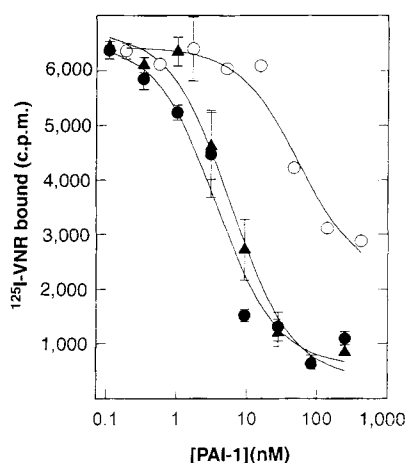
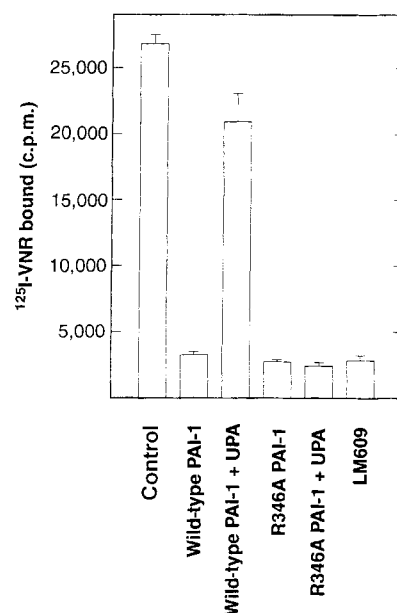


FIG. 2 Inhibition of ¹²⁵I-VNR binding to vitronectin by PAI-1 with and without UPA. ¹²⁵I-VNR bound to vitronectin is shown in the presence of each competitor. Data represent the average of 2 experiments performed in duplicate.



LM609, a monoclonal antibody to $\alpha_v\beta_3$, also inhibits the binding of ^{125}I -VNR to vitronectin to the same extent as PAI-1 (Fig. 2), confirming that the VNR preparation contains primarily $\alpha_v\beta_3$, and that PAI-1 can block the binding of this integrin to vitronectin.

To see whether PAI-1 inhibits the interaction of cellular integrins with vitronectin in a similar manner, attachment and migration assays using SMCs were performed. Both active wild-type PAI-1 and mutant R346A PAI-1 inhibited SMC attachment to vitronectin (Fig. 3a). In contrast, the mutant Q123K PAI-1 had no effect on cell attachment, indicating that the inhibition of cell attachment by PAI-1 is due to its ability to bind vitronectin, and not its inhibitory activity towards plasminogen activators. Furthermore, adding UPA to wild-type PAI-1 and vitronectin reversed the inhibition of cell attachment, whereas UPA had no effect on the inhibition of SMC attachment by R346A PAI-1. PAI-1 was found to inhibit the attachment of SMCs to vitronectin, whereas LM609 is not, suggesting that SMCs have other integrins that can mediate attachment to vitronectin through the RGD integrin binding site²⁰, and that PAI-1 blocks access of these integrins as well. Consistent with this interpretation, a synthetic RGD-containing peptide also blocked SMC attachment to vitronectin (data not shown).

PAI-1 also inhibited the migration of SMC through vitronectin-coated Transwells. As observed with cell attachment, both active wild-type PAI-1 and R346A PAI-1 prevented cell migration, whereas active Q123K PAI-1 had no effect (Fig. 3b). Inclusion of UPA negated the inhibition of migration by wild-type PAI-1 but not by R346A PAI-1. This demonstrates that, like attachment, the inhibition of cell migration results from the capacity of PAI-1 to bind vitronectin, and not its inhibitory activity towards plasminogen activators. LM609, which did not prevent attachment, inhibited migration, confirming that SMCs require $\alpha_v\beta_3$ for motility¹¹. These data are consistent with observations that migrating vascular cells show increased expression of both UPA^{2,9} and the VNR¹, and that SMCs show greater migration on vitronectin than on other matrix proteins¹¹.

Thus SMC migration during normal wound healing requires both the cellular expression of $\alpha_v\beta_3$ and the presence of vitronectin in the matrix, and our data suggest that PAI-1 might be an important regulator of this process. However, subcellular matrices *in vivo* are much more complex, containing collagens, glycosaminoglycans and other proteins, such as laminin. Therefore, to examine the role of PAI-1 and vitronectin in cellular attachment and migration on such a heterogeneous matrix, and to see if PAI-1 can regulate this process, attachment and migration assays were performed on a complex basement-membrane matrix derived from murine sarcoma cells (Matrigel) with and without vitronectin. Unlike cell attachment to purified vitronectin, PAI-1 had no effect on attachment to Matrigel even in the presence of vitronectin (data not shown). This indicates that other proteins present in the matrix can support cell attachment and, as was seen with fibronectin (data not shown), PAI-1 had no effect on this association. PAI-1 also had no effect on SMC migration through Matrigel-coated Transwells in the absence of added vitronectin. However, adsorption of vitronectin to the Matrigel markedly increased SMC migration (Fig. 4). This is consistent with previous reports demonstrating that vitronectin significantly enhances migration of both SMCs^{21,22} and primary keratinocytes²³, and suggests that the

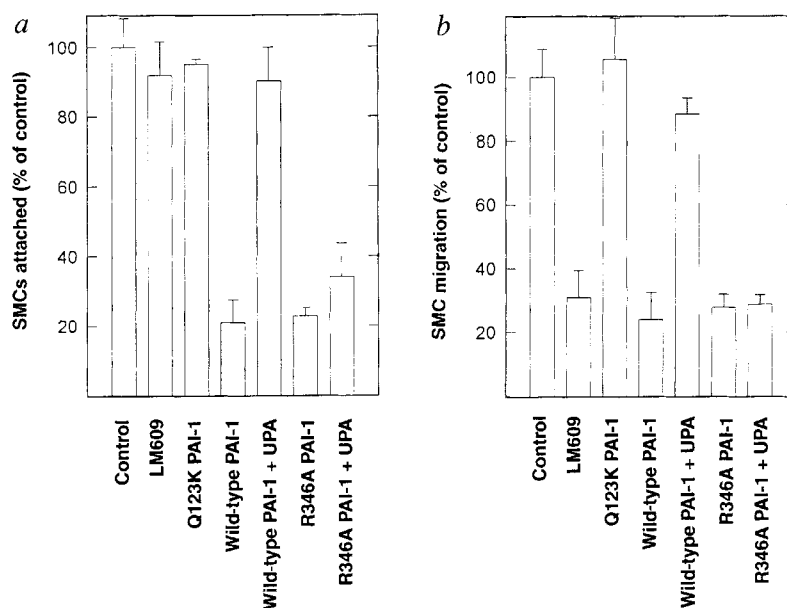


FIG. 3 Attachment and migration of rabbit SMCs on vitronectin. *a*, Amount of cell attachment to vitronectin-coated plates in the presence of each competitor. *b*, Extent of cell migration through vitronectin-coated Transwells in the presence of each competitor. Data represent the average of 5 experiments (*a*) or 3 experiments (*b*), all performed in duplicate.

presence of vitronectin in an exposed matrix or fibrin clot might stimulate cell migration. Furthermore, even though PAI-1 and LM609 had no effect on cell attachment to Matrigel containing vitronectin, they inhibited cell migration on this complex matrix (Fig. 4). As with purified vitronectin, the Q123K PAI-1 had no effect on cell migration, and the addition of UPA reversed the inhibition by wild-type PAI-1. Together, these data demonstrate that SMC migration on a complex matrix is enhanced by adsorption of vitronectin, and that this induction is $\alpha_v\beta_3$ dependent. PAI-1 can prevent this induction by blocking $\alpha_v\beta_3$ binding, and plasminogen activators can promote the induction by reversing the PAI-1 block. This suggests that *in vivo* the binding of plasma vitronectin to an exposed matrix after injury may accelerate cell migration during wound healing, and that PAI-1 may be an important regulator of this process. Supporting this hypothesis, treatment of Matrigel-coated Transwells with bovine serum enhanced SMC migration in a PAI-1 inhibitable manner (data not shown). These results are also consistent with the observation that PAI-1 null mice show enhanced SMC migration and proliferation, whereas in UPA null mice SMC migration is reduced¹².

The specificity of the inhibition of integrin attachment to vitronectin by active PAI-1 further illustrates the unique functional interdependence that exists between PAI-1 and vitronectin. As well as stabilizing PAI-1 in the active conformation^{18,24}, vitronectin also alters the specificity of PAI-1, making it an efficient inhibitor of thrombin^{25,26}, and promoting its clearance by members of the LDL receptor family¹⁹. This suggests that thrombin, a known mitogen and chemotactant, might also promote cell migration by removing PAI-1 from vitronectin. Several previous studies have shown that both elastase and cathepsin G produced by activated neutrophils can efficiently remove PAI-1 from the matrix^{27,28}. Because PAI-1 is a substrate for these enzymes, only amounts necessary for catalysis are required to inactivate PAI-1. Together these data indicate that a wide variety of proteinases can interact with PAI-1 and expose the RGD integrin binding site on vitronectin. This general ability to modify cellular adhesive properties by many divergent proteinases through a common mechanism suggests that the known correlation between increased cell migration

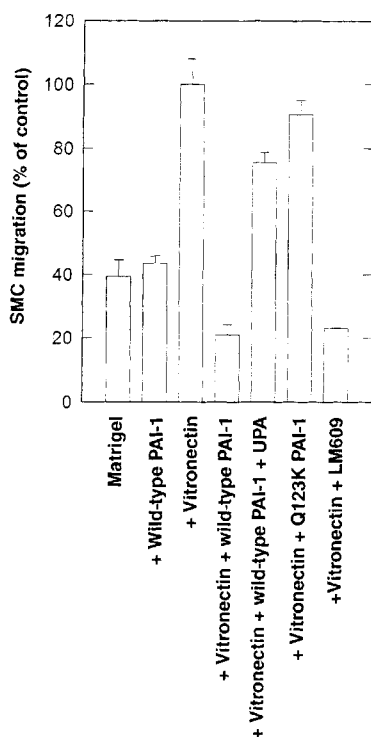


FIG. 4 Migration of rabbit SMCs through Matrigel-coated Transwells with and without vitronectin. The extent of cell migration through Matrigel-coated Transwells is shown with and without vitronectin in the presence of each competitor. The data represent the average of 3 experiments performed in duplicate.

and proteinase activity may be mediated, at least in part, by proteolytic interaction with PAI-1 in a wide variety of invasive cellular processes. It also suggests that the role of some proteinases in cellular migration may not be to cause generalized matrix degradation, but rather may be to expose cryptic cell-attachment sites by inactivating PAI-1. □

Methods

PAI-1 competition of ¹²⁵I-VNR binding to vitronectin. Active forms of wild-type PAI-1 (Molecular Innovations) and PAI-1 mutants were prepared²⁹. Native vitronectin (Molecular Innovations) was coated onto microtitre wells (1 µg ml⁻¹) for 2 h at 37 °C, followed by blocking with 2% BSA in TBS, 5 mM CaCl₂. VNR was purified from human placenta³⁰. Radiolabelled VNR (2.5 nM) was allowed to bind vitronectin in the presence of increasing concentrations of wild-type PAI-1 or PAI-1 mutants. For analysis with and without UPA, 500 nM PAI-1 was allowed to bind to vitronectin as above, unbound PAI-1 was removed, and 5 nM ¹²⁵I-VNR added either alone or in the presence of 1 µM UPA or 50 µg ml⁻¹ LM609 (Chemicon). Assays were processed and analysed as described¹⁹.

Attachment and migration of rabbit SMC on vitronectin. Washed cells were resuspended in serum-free media with and without 500 nM of wild-type PAI-1, Q123K PAI-1 or R346A PAI-1, either alone or with 1 µM UPA or with LM609 (0.5 µg ml⁻¹) alone. Cells were allowed to attach to vitronectin-coated plates (1 µg ml⁻¹) for 30 min, then washed twice with TBS, fixed in methanol/acetic acid (75/25 v/v), and stained with 2% crystal violet. Absorbance of stained cells was measured using a Sony CCD/RGB colour video system with image-1 software (Universal Imaging). Cell attachment to vitronectin alone was established as 100%, and the attachment of each experimental condition was calculated as a ratio to this value. Analysis of known cell numbers treated similarly indicated that absorbance was linear over the range of cells examined. Migration assays were performed on Transwells (3-µm pore size) coated with vitronectin. SMCs were allowed to attach and spread for 45–60 min on the upper chamber in serum-free media. LM609 (0.5 µg ml⁻¹) or 1 µM wild-type PAI-1 or PAI-1 mutants with and without 2 µM UPA were added in 0.5% BSA, 1 mM CaCl₂, 0.5 mM MnCl₂ and Nutridoma (Boehringer). After 4–5 h the upper cell layer was removed with a cotton swab, and cells on the underside of the Transwell were fixed, stained and analysed as above, with the amount of cell migration observed with vitronectin alone established as 100%. Migration on Matrigel with and without vitronectin was as above, except that Transwells were first coated with Matrigel (1:20 dilution) in serum-free media overnight at 4 °C, followed by washing with PBS and blocking with 1% BSA in PBS. Transwells were then incubated with and without vitronectin (0.2 mg ml⁻¹) followed by washing with PBS before addition of cells. Migration was determined after 14–16 h incubation.

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CORRESPONDENCE and requests for materials should be addressed to D.A.L. (e-mail: lawrencd@usa.redcross.org).

TRAF6 is a signal transducer for interleukin-1

Zhaodan Cao, Jessie Xiong, Masahiro Takeuchi*, Takeshi Kurama* & David V. Goeddel

Tularik Inc., Two Corporate Drive, South San Francisco, California 94080, USA

* Yamanouchi Pharmaceutical Co. Ltd, 21 Miyukigaoka, Tsukuba, Ibaraki 305, Japan

MANY cytokines signal through different cell-surface receptors to activate the transcription factor NF-κB (ref. 1). Members of the TRAF protein family have been implicated in the activation of NF-κB by the tumour-necrosis factor (TNF)-receptor superfamily^{2–5}. Here we report the identification of a new TRAF family member, designated TRAF6. When overexpressed in human 293 cells, TRAF6 activates NF-κB. A dominant-negative mutant of TRAF6 inhibits NF-κB activation signalled by interleukin-1 (IL-1) but not by TNF. IL-1 treatment of 293 cells induces the association of TRAF6 with IRAK, a serine/threonine kinase that is rapidly recruited to the IL-1 receptor after IL-1 induction⁶. These findings indicate that TRAF proteins may function as signal transducers for distinct receptor families and that TRAF6 participates in IL-1 signalling.

The transcription factor NF-κB is activated by many pro-inflammatory and immunomodulatory molecules, including IL-1, TNF, lipopolysaccharide (LPS), CD30 and CD40 ligands, lymphotoxin-α (LT-α) and LT-β (refs 1, 4, 7–10). CD30, CD40, and receptors for TNF, LT-α and LT-β belong to the TNF-receptor superfamily¹¹, whereas the receptors for IL-1 and LPS

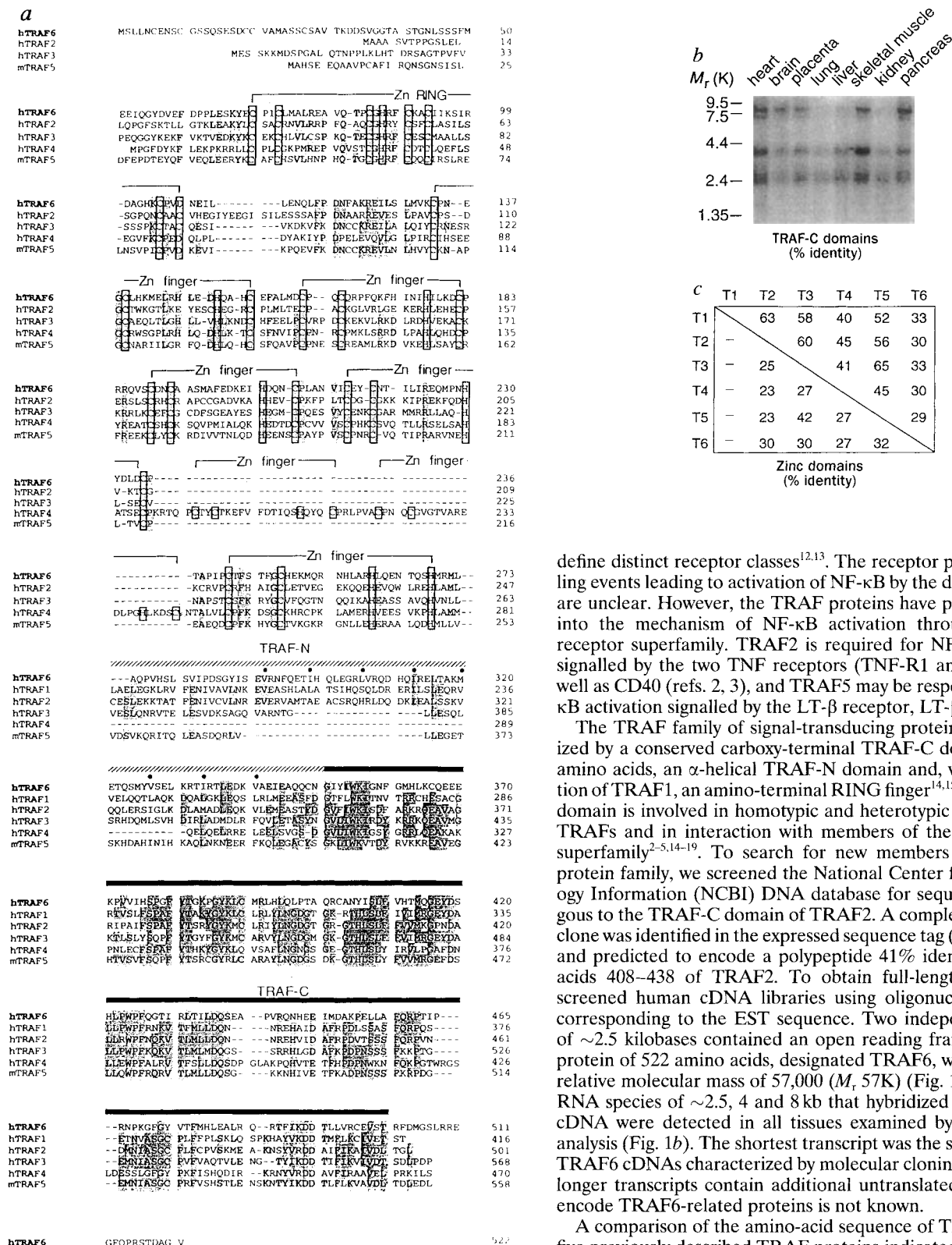


FIG. 1 Sequence comparison of TRAFs and tissue distribution of TRAF6 mRNA. **a**, Sequence alignment of human TRAF1, 2, 3, 4, 6 and mouse TRAF5 proteins^{4,14,15,17,28}. Residues critical for the formation of the N-terminal RING and zinc-finger structures are boxed. The periodically occurring hydrophobic residues in the TRAF-N domain, indicative of a coiled-coil, are marked with dots. **b**, Northern-blot analysis to detect TRAF6 mRNA in human tissues. RNA size markers are indicated in kb. **c**, Pairwise comparison showing the degree of sequence identity shared among the six TRAF proteins in the TRAF-C and zinc-binding domains.

define distinct receptor classes^{12,13}. The receptor proximal signalling events leading to activation of NF- κ B by the different ligands are unclear. However, the TRAF proteins have provided insight into the mechanism of NF- κ B activation through the TNF-receptor superfamily. TRAF2 is required for NF- κ B activation signalled by the two TNF receptors (TNF-R1 and TNF-R2) as well as CD40 (refs. 2, 3), and TRAF5 may be responsible for NF- κ B activation signalled by the LT- β receptor, LT- β R (ref. 4).

The TRAF family of signal-transducing proteins is characterized by a conserved carboxy-terminal TRAF-C domain of ~170 amino acids, an α -helical TRAF-N domain and, with the exception of TRAF1, an amino-terminal RING finger^{14,15}. The TRAF-C domain is involved in homotypic and heterotypic aggregation of TRAFs and in interaction with members of the TNF-receptor superfamily^{2-5,14-19}. To search for new members of the TRAF protein family, we screened the National Center for Biotechnology Information (NCBI) DNA database for sequences homologous to the TRAF-C domain of TRAF2. A complementary DNA clone was identified in the expressed sequence tag (EST) database and predicted to encode a polypeptide 41% identical to amino acids 408–438 of TRAF2. To obtain full-length cDNAs, we screened human cDNA libraries using oligonucleotide probes corresponding to the EST sequence. Two independent cDNAs of ~2.5 kilobases contained an open reading frame encoding a protein of 522 amino acids, designated TRAF6, with a predicted relative molecular mass of 57,000 (M_r , 57K) (Fig. 1a). Messenger RNA species of ~2.5, 4 and 8 kb that hybridized to the TRAF6 cDNA were detected in all tissues examined by northern blot analysis (Fig. 1b). The shortest transcript was the same size as the TRAF6 cDNAs characterized by molecular cloning. Whether the longer transcripts contain additional untranslated sequences or encode TRAF6-related proteins is not known.

A comparison of the amino-acid sequence of TRAF6 with the five previously described TRAF proteins indicated a high degree of structural similarity (Fig. 1a). The C-terminal 160 amino acids of TRAF6 share ~30% sequence identity with the TRAF-C domains of TRAFs 1–5, making TRAF6 the most distantly related TRAF family member yet described (Fig. 1a,c). Amino acids 274–350 of TRAF6, while unrelated to the other TRAFs in primary sequence, constitute a TRAF-N domain, as they have the potential to form an amphipathic α -helix. The cysteine-rich N terminus of TRAF6 (amino acids 70–273) includes a RING finger and five potential zinc-finger structures. TRAFs 2, 3 and 5 contain these same features, whereas TRAF4 contains two additional

zinc-fingers. The overall sequence identity between TRAF6 and TRAFs 2–5 in the presumptive zinc-binding domains is approximately 30% (Fig. 1c).

The previously described TRAF proteins are all able to homodimerize through the TRAF domain^{4,14–16,18}. Furthermore, TRAF1 and TRAF2 form a heterotypic complex^{5,14}. Yeast two-hybrid and mammalian cell co-immunoprecipitation assays indicate that TRAF6 interacts strongly with itself, and weakly with both TRAF2 and TRAF3 (data not shown). Therefore, TRAF6 is likely to associate either with itself or with as yet unidentified partners.

TRAF2 and TRAF5 have been implicated in NF- κ B activation mediated by members of the TNF-receptor family^{2–5}. Overexpression of either TRAF2 or TRAF5 in cells results in NF- κ B activation. To determine whether TRAF6 might also play a role in NF- κ B activation, we used electrophoretic-mobility-shift and reporter-gene assays in transiently transfected cells. Nuclear extracts of TRAF6-overexpressing 293 cells contained a significant amount of NF- κ B DNA-binding activity (Fig. 2a). Overexpression of TRAF6 also led to activation of a co-transfected NF- κ B-dependent reporter gene (Fig. 2b), but TRAF6 failed to activate a control construct² in which the NF- κ B sites were mutated (data not shown).

To identify the structural domain(s) of TRAF6 responsible for this activity, we tested a series of deletion mutants for activation of an NF- κ B-dependent reporter gene (Fig. 2c). TRAF6 lacking the RING finger was active in this assay, but the activity was greatly reduced in the TRAF6 (184–522) mutant, in which zinc-fingers 1 and 2 were also deleted. TRAF6 mutants lacking either the entire zinc-binding region or zinc-fingers 3–5 failed to activate NF- κ B. TRAF6 may therefore activate NF- κ B by a mechanism distinct from TRAF2 and TRAF5, both of which require an intact RING finger to trigger NF- κ B activation^{2,4}.

The observed weak interaction between TRAF2 and TRAF6 indicated that TRAF6 might be involved in TNF-mediated NF- κ B activation. Therefore, we tested whether a TRAF6 polypeptide lacking the N-terminal zinc-binding structures could act as a dominant-negative mutant to block NF- κ B activation signalled by TNF. TRAF6(289–522) overexpression (Fig. 3a) had minimal

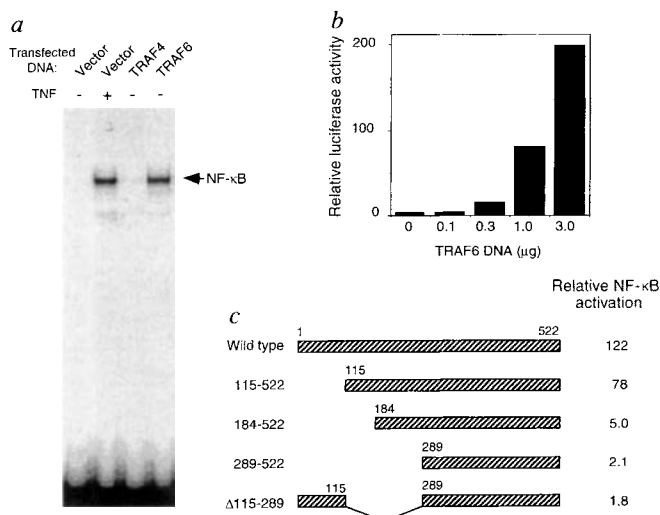


FIG. 2 NF- κ B activation induced by TRAF6. **a**, EMSA demonstrating NF- κ B DNA-binding activity in 293 cells treated with TNF or overexpressing TRAF6. **b**, Activation of NF- κ B by TRAF6 in 293 cells as measured by reporter gene activity. **c**, Delineation of the region of TRAF6 required for NF- κ B activation. The portions of TRAF6 expressed are shaded. The ability of these truncated proteins to activate NF- κ B was tested by the reporter assay. Relative NF- κ B activation was calculated by dividing the luciferase activity determined in cells transfected with the indicated expression plasmids by that determined in cells transfected with an empty vector.

effect on TNF signalling under conditions in which overexpression of a TRAF2 dominant-negative mutant, TRAF2(87–501) (ref. 2), completely blocked TNF-mediated NF- κ B activation. These results indicate that TRAF6 may not be involved in TNF signalling.

Using the same approach, we tested whether TRAF6 might participate in IL-1 signal transduction. A 293 cell line, which stably overexpresses the type I IL-1 receptor (293 IL-1RI), responded to IL-1 treatment with an ~160-fold activation of an NF- κ B-dependent reporter gene. When these cells were transfected with an expression vector for TRAF6(289–522), IL-1-dependent NF- κ B activation was inhibited in a dose-dependent manner (Fig. 3b). Overexpression of TRAF2(87–501) did not affect NF- κ B activation signalled by IL-1, demonstrating that the dominant-negative effect of the TRAF6 mutant was specific. Similarly, overexpression of truncated TRAF5 did not inhibit IL-1-mediated NF- κ B activation (data not shown). Therefore, TRAF6 is likely to be a component of the signalling cascade that starts at the IL-1 receptor and results in the activation of NF- κ B.

We have previously identified a Ser/Thr kinase, designated IRAK, that is rapidly recruited to the type I IL-1 receptor (IL-1RI) complex and becomes autophosphorylated after IL-1 treatment⁶. IRAK is thought to be a mammalian homologue of Pelle, a *Drosophila melanogaster* protein kinase required for the activation of the *D. melanogaster* NF- κ B homologue Dorsal²⁰. To investigate whether TRAF6 might interact with IL-1RI and/or IRAK, 293 IL-1RI cells were transfected with expression vectors encoding Flag-epitope-tagged TRAF2 and TRAF6 proteins, and treated briefly with IL-1. Immunoprecipitation of IL-1RI did not coprecipitate either TRAF2 or TRAF6, and vice versa (data not shown), indicating that neither TRAF is stably recruited to the IL-1 receptor complex. However, immunoprecipitation of the TRAF

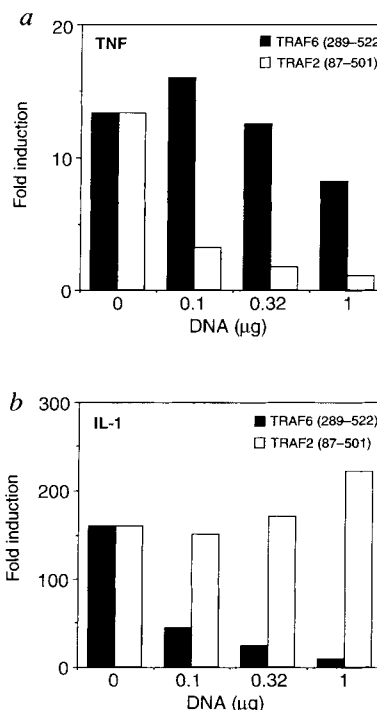
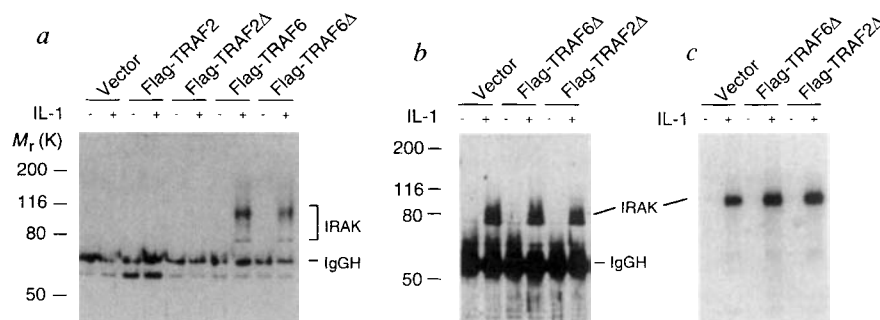


FIG. 3 Selective inhibition of IL-1-induced NF- κ B activity by TRAF6(289–522). **a**, Lack of effect of TRAF6(289–522) on TNF-induced NF- κ B activity. Luciferase activity was determined in 293 cells transfected with expression plasmids for either TRAF6(289–522) or TRAF2(87–501) together with the ELAM-1 luciferase reporter plasmid²⁶ for 24 h, followed by 6 h of TNF (10 ng ml⁻¹) treatment. **b**, Inhibition of IL-1-induced NF- κ B activity by overexpressing TRAF6(289–522). 293 cells were transfected as for **a**, but were treated with IL-1 (10 ng ml⁻¹) for 6 h before measurement of luciferase activity.

FIG. 4 Interaction of TRAF6 with IRAK in transfected cells. **a**, IL-1-dependent association of TRAF6 with IRAK. Immunoblot analysis was used to detect IRAK in the TRAF complexes precipitated with an anti-Flag antibody from IL-1-treated (+) or untreated (-) 293 IL-1RI cells⁶ expressing the indicated TRAF proteins. TRAF2(87–501) and TRAF6(289–522) are labelled as TRAF2Δ and TRAF6Δ, respectively. **b**, Lack of effect of TRAF6(289–522) on IL-1-induced interaction between IRAK and IL-1RI. IRAK was detected by immunoblotting in the IL-1RI complex isolated from IL-1-treated cells overexpressing TRAF6(289–522) or TRAF2(87–501). **c**, Lack of effect of TRAF6(289–522) on IRAK autophosphorylation in the IL-1RI complex. IL-1RI immunoprecipitants from transfected 293 IL-1RI cells



(as in b) were incubated in kinase buffer with [γ -³²P]-ATP, and then resolved by SDS-PAGE. Abbreviation: IgGH, immunoglobulin G heavy chain.

proteins demonstrated that IRAK associates with both full-length and truncated TRAF6 in IL-1-treated cells (Fig. 4a). IRAK did not coprecipitate with TRAF6 in untreated cells. Moreover, IRAK did not associate with the full-length or truncated TRAF2, even when cells were treated with IL-1. These observations further implicate TRAF6, but not TRAF2, in IL-1 signalling.

We next investigated whether the dominant-negative mutant TRAF6(289–522) blocks IL-1 signalling by interfering with either the autophosphorylation of IRAK, or its interaction with IL-1RI. Similar amounts of IRAK were detected in IL-1RI complexes from cells overexpressing either TRAF6(289–522) or TRAF2(87–501) (Fig. 4b). Similarly, IRAK autophosphorylation was unaffected by overexpression of TRAF6(289–522) (Fig. 4c). These results indicate that the role of TRAF6 in IL-1 signal transduction might be to connect IRAK with a downstream signalling cascade. If so, TRAF6(289–522) may disrupt this IL-1 signalling pathway by altering the ability of IRAK to recognize a downstream substrate. This sequential order of events is consistent with our inability to detect TRAF6 in the IL-1RI complex. Detection of TRAF6 in complex with IRAK, but not with IL-1RI, suggests that IRAK only transiently associates with IL-1RI after IL-1 induction.

The TRAFs represent an expanding family of signal-transducing proteins, with three of the six described members, TRAF2, TRAF5 and TRAF6, being involved in NF- κ B activation. Evidence that TRAF6 is a component of the IL-1 signalling pathway extends the role of TRAFs beyond being signal transducers for the TNF-receptor superfamily. TRAF2 protein interacts with several signalling molecules, including TRADD, RIP and cIAPs (refs 3, 5, 22, 22). The repertoire of TRAF complexes that can be integrated into different levels of signalling cascades is further expanded by the ability of TRAFs to form homo- and hetero-oligomers. For example, TRAFs participate in signalling through TNF-R2, CD40 and LT- β R by binding directly to the receptors, and in signalling through TNF-R1 by binding to the adaptor molecule TRADD (refs 2–4, 14). Although the exact role of TRAFs in NF- κ B activation is unknown, TRAF2 interacts with RIP, a Ser/Thr kinase implicated in NF- κ B-activation signalled by TNF (ref. 21). We now show ligand-dependent interaction of TRAF6 with IRAK. It is therefore possible that the function of TRAF proteins is to regulate specific activities of certain protein kinases. Understanding how the interaction between TRAF6 and IRAK contributes to NF- κ B activation will help to elucidate the role of TRAF proteins as mediators of diverse extracellular signals. □

Methods

Construction of expression plasmids. The expression plasmids for TRAF4 and TRAF6 were constructed by inserting the coding sequences into a mammalian expression plasmid pRK5 (ref. 23), downstream of the cytomegalovirus immediate-early promoter and enhancer. The TRAF6 truncation mutants were generated by inserting TRAF6 cDNA fragments amplified by polymerase chain reaction into pRK5.

Electrophoretic mobility shift assay (EMSA). 2×10^6 293 cells were plated in 10-cm dishes and transfected on the following day with 5 μ g of the indicated plasmid DNA by calcium phosphate precipitation²⁴. After the transfection (24 h), nuclear extracts were prepared for EMSA²⁵. The oligonucleotide used for EMSA contained the sequence 5'-GATGCCATTGGGATTCCTCTTACTG-3', which was derived from the human ELAM-1 promoter²⁶.

ELAM-1 reporter assay. 2×10^5 293 cells were plated in 3.5-cm dishes and transfected on the following day with indicated amounts of expression plasmids, 1 μ g ELAM-1-luciferase reporter plasmid²⁶ and 0.2 μ g of plasmid containing the β -galactosidase gene controlled by the cytomegalovirus promoter to control for transfection efficiency. The total amount of DNA was kept constant in all transfections by adding pRK5 vector DNA. The cells were lysed for measurement of luciferase activity using reagents from Promega.

Co-immunoprecipitation and *in vitro* kinase assays. 5×10^6 293 IL-1RI cells were plated on 15-cm dishes and transfected with 6 μ g of the indicated expression plasmid. Estimated transfection efficiencies for 293 cells were 50–95% (data not shown). After transfection (38 h), cells were collected, induced with IL-1 (200 ng ml⁻¹) for 5 min and lysed⁶. TRAF proteins were precipitated with 3 μ g M2 monoclonal antibody to the Flag epitope (VWR), and 10 μ l of protein-G-agarose, then immunoblotted with polyclonal antiserum to IRAK. Proteins were detected with ECL reagent (Amersham). To detect IRAK associated with the IL-1 receptor, the IL-1RI complex was precipitated with polyclonal antibody to IL-1RI (ref. 27), and 10 μ l protein-A-agarose, and IRAK detected by immunoblotting. To assess the ability of IRAK to autophosphorylate, the IL-1RI immunocomplex was incubated in 20 μ l kinase buffer²⁷ with 10 μ Ci [γ -³²P]ATP for 30 min at 30 °C and analysed by SDS-PAGE followed by autoradiography.

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CORRESPONDENCE and requests for materials should be addressed to D.V.G. (e-mail: goeddel@tulink.com). The GenBank accession number for the human TRAF6 is L81153. The TRAF6 cDNA clone was identified from the Washington University-Merck EST project, with accession number T82812.

β -Arrestin acts as a clathrin adaptor in endocytosis of the β_2 -adrenergic receptor

Oscar B. Goodman, Jr, Jason G. Krupnick, Francesca Santini, Vsevolod V. Gurevich*, Raymond B. Penn, Alison W. Gagnon, James H. Keen & Jeffrey L. Benovic

Department of Biochemistry and Molecular Pharmacology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, USA

THE ability of a system to regulate its responsiveness in the presence of a continuous stimulus, often termed desensitization, has been extensively characterized for the β_2 -adrenergic receptor (β_2 AR). β_2 AR signalling is rapidly attenuated through receptor phosphorylation and subsequent binding of the protein β -arrestin^{1,2}. Ultimately the receptor undergoes internalization^{3,4}, and although the molecular mechanism is unclear, receptor phosphorylation and β -arrestin binding have been implicated in this process^{5,6}. Here we report that β -arrestin and arrestin-3, but not visual arrestin, promote β_2 AR internalization and bind with high affinity directly and stoichiometrically to clathrin, the major structural protein of coated pits. Moreover, β -arrestin/arrestin chimaeras that are defective in either β_2 AR or clathrin binding show a reduced ability to promote β_2 AR endocytosis. Immunofluorescence microscopy of intact cells indicates an agonist-dependent colocalization of the β_2 AR and β -arrestin with clathrin. These results show that β -arrestin functions as an adaptor in the receptor-mediated endocytosis pathway, and suggest a general mechanism for regulating the trafficking of G-protein-coupled receptors.

As internalization of β_2 AR appears to occur through clathrin-coated pits⁷, we considered that arrestins might function to target β_2 AR to clathrin-coated pits. To test this, we examined whether arrestins interact with clathrin, the major structural protein of coated pits. *In vitro* translated β -arrestin and arrestin-3 bound specifically to clathrin cages, whereas visual arrestin did not (Fig. 1a, b). Short and long polypeptide splice variants of β -arrestin and arrestin-3 (ref. 8) bound similarly to clathrin cages (data not shown).

To determine whether β -arrestin and arrestin-3 interact directly with clathrin, we assessed the binding of purified recombinant arrestins to assembled clathrin structures (Fig. 2a). Purified β -arrestin and arrestin-3 bound specifically and with high affinity to pure clathrin cages at pH 6.8, with only a small fraction sedimenting nonspecifically (Fig. 2b, compare lanes 2 and 3). Half-maximal binding occurred at 64 nM and 10 nM clathrin, respectively (Fig. 2c), comparable to that of AP-2 protein binding to clathrin ($K_d \sim 10$ nM)^{9,10}. To investigate arrestin binding at physiological pH, we used lightly crosslinked clathrin cages¹¹ (Fig. 2a, lane 4) or clathrin-coated vesicles (CVs) (Fig. 2a, lane 5), the latter a model for an intact plasma-membrane coated-pit. Both β -arrestin and arrestin-3 bound to these clathrin substrates comparably (Fig. 2b, lanes 4 and 5). By varying the concentration of arrestin-3, the stoichiometry was determined to be 3–4 mol arrestin bound per mol sedimented clathrin (Fig. 2d). This is consistent with the structure of clathrin containing three heavy and three light chains. Arrestin binding was rapid ($t_{1/2} < 1$ min) and also occurred with dissociated clathrin trimers immobilized on Sepharose (data not shown). Together with the fact that arrestins interact with the β_2 AR (refs 1, 12), these results provide evidence for a clathrin–arrestin interaction and its role in the β_2 AR internalization process.

We next localized the clathrin binding domain by assessing the ability of two β -arrestin/arrestin chimaeras, whose β_2 AR binding

has been characterized¹², to bind to clathrin cages (Fig. 3). The chimaera AAAB (residues 1–345 of visual arrestin and 341–418 of β -arrestin) bound to clathrin cages almost as well as did β -arrestin ($68 \pm 9\%$), indicating that the C-terminal ~ 77 amino acids of β -arrestin are important in the interaction with clathrin. The C terminus is a very acidic domain that shows significant similarity between β -arrestin and arrestin-3 (58% identity), but not between β -arrestin and arrestin (27% identity). The chimaera ABBA (residues 1–47 and 346–404 of arrestin and 44–340 of β -arrestin) bound relatively weakly to clathrin cages ($33 \pm 4\%$), although significantly better than did arrestin ($6 \pm 1\%$). This suggests that the C-terminal 77 amino acids comprise the major clathrin-binding domain in β -arrestin, but additional residues may contribute.

ABBA has reduced clathrin and normal β_2 AR binding¹², whereas AAAB has reduced receptor binding¹² with significant retention of clathrin binding. We were therefore able to assess the relative roles of these domains in promoting β_2 AR internalization. Cells transfected with β_2 AR alone or co-transfected with β_2 AR and visual arrestin had a 15% loss of cell-surface receptors after treatment with the agonist isoprenaline (Fig. 3). Arrestin is therefore unable to enhance β_2 AR internalization, consistent with its weak binding to β_2 AR (ref. 12) and to clathrin (Fig. 1). By contrast, cells coexpressing β_2 AR and β -arrestin lost 40% of their cell surface β_2 ARs after agonist treatment, indicating that β -arrestin augments β_2 AR internalization. If both the β_2 AR- and clathrin-binding properties of β -arrestin are critical in promoting receptor internalization, both chimaeras should have a reduced ability (relative to β -arrestin) to promote β_2 AR internalization. Indeed, in COS-1 cells, ABBA was only $\sim 30\%$, and AAAB $\sim 10\%$, as efficient as β -arrestin in promoting β_2 AR internalization, supporting a critical role for the clathrin- and receptor-binding domains of β -arrestin in mediating β_2 AR internalization.

The ability of β -arrestin and arrestin-3 to bind both G-protein-coupled receptors¹² and clathrin (Figs 1 and 2) suggests that non-visual arrestins function as adaptor proteins to promote receptor localization in clathrin-coated pits. We therefore asked whether β_2 ARs in intact cells are localized in clathrin-coated pits in an agonist- and β -arrestin-dependent manner. Cell surface Flag-tagged β_2 ARs were visualized by labelling fixed, unpermeabilized

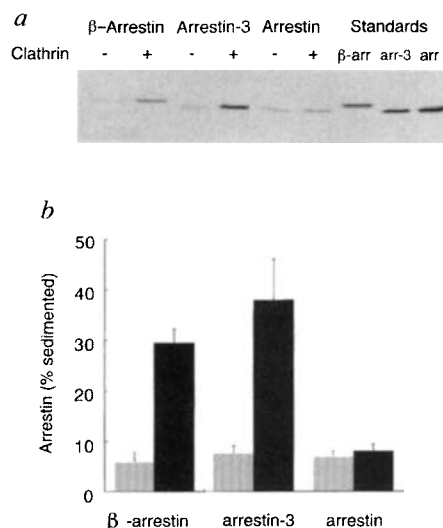


FIG. 1 Binding of *in vitro*-translated arrestins to clathrin cages. **a**, *In vitro*-translated β -arrestin, arrestin-3, and visual arrestin (0.5–1.0 nM) were incubated in the absence (–) or presence (+) of clathrin cages (200 nM), centrifuged, and analysed as described in Methods. The standards represent 50% of the total arrestin input. **b**, Quantification of *in vitro*-translated arrestin binding to clathrin cages. Black bars represent arrestin sedimentation in the presence of cages; hatched bars represent sedimentation in the absence of cages. Data are expressed as the percentage of total arrestin sedimented and are the mean $\pm$ s.e.m. of four independent experiments.

* Present address: Sun Health Research Institute, Sun City, Arizona 85372, USA.

cells with anti-Flag antibodies, and clathrin was detected in the same cells after permeabilization. In the absence of agonist, β_2 ARs gave a diffuse signal on the cell surface, while there was a characteristic peripheral punctate pattern of plasma membrane clathrin-coated pits and perinuclear staining in the Golgi region¹³ (data not shown). However, following a 10 min incubation with isoprenaline, surface β_2 ARs were distributed in a punctate manner and were largely coincident with plasma-membrane clathrin-coated pits (Fig. 4a,b). Although the relative intensity

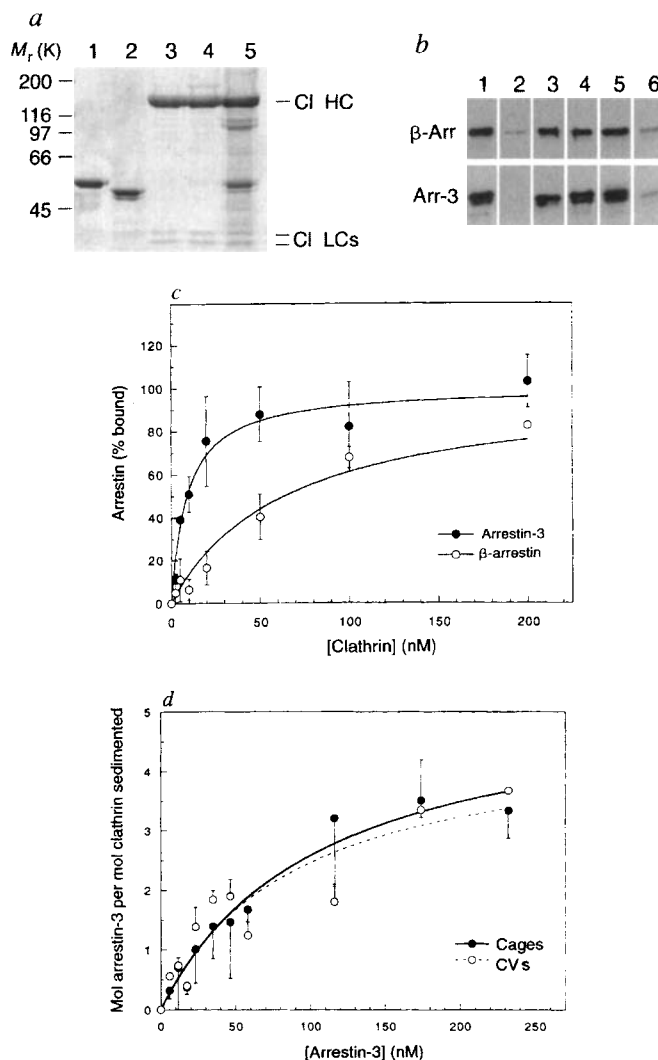


FIG. 2 Binding of purified recombinant β -arrestin and arrestin-3 to clathrin cages and coated vesicles. **a**, 3 μ g each of β -arrestin (lane 1), arrestin-3 (lane 2), intact clathrin cages (lane 3), crosslinked clathrin cages (lane 4), and CVs (lane 5; 3 μ g clathrin trimers) were electrophoresed and stained with Coomassie blue. Molecular mass markers ($M_r \times 10^{-3}$) are to the left, and positions of the clathrin heavy (CI HC) and light chains (CI LCs) are indicated on the right. **b**, Binding of arrestins to intact clathrin cages, crosslinked cages and CVs. Arrestins (150 nM) without (lane 2) or with intact clathrin cages (lane 3), crosslinked clathrin cages (lane 4) or CVs (lane 5) (50 nM clathrin trimers for each) were incubated and then processed by centrifugation, electrophoresis and immunoblotting. Lane 1 denotes 50% of the arrestin input; lane 6 is 100% of the endogenous arrestins in the CV preparation. **c**, β -arrestin or arrestin-3 (15 nM) was incubated with increasing concentrations of clathrin cages (0–200 nM clathrin) in buffer A containing 0.1 mg ml⁻¹ bovine serum albumin and then processed by centrifugation, electrophoresis, and immunoblotting. Data shown are the mean $\pm$ s.e.m. of three independent experiments. **d**, CVs and crosslinked cages (5 nM clathrin trimers) were incubated with increasing concentrations of arrestin-3 (0–235 nM) and then processed by centrifugation, electrophoresis and immunoblotting. Data shown are the mean $\pm$ s.e.m. of three independent experiments.

of the signals varied, each of the β_2 AR dots appeared coincident with a clathrin dot, demonstrating that a substantial proportion of the surface β_2 ARs localize in clathrin-coated pits in an agonist-dependent manner. Agonist-dependent colocalization of surface β_2 AR with clathrin required coexpression of β -arrestin. Incubation of agonist-treated cells with fluorescently labelled transferrin revealed punctate colocalization of Flag-tagged β_2 AR and transferrin, indicating that internalized β_2 AR is delivered to an early endosomal compartment (data not shown).

Because β -arrestin is thought to associate with the agonist-occupied phosphorylated β_2 AR, we investigated whether β -arrestin and the β_2 AR were colocalized. COS-1 cells coexpressing β -arrestin and Flag-tagged β_2 AR were incubated in the presence or absence of isoprenaline for 10 min. Total β -arrestin was diffusely distributed before addition of agonist (Fig. 4c and d), whereas after the addition of agonist, a distinct punctate pattern in which virtually all surface β_2 AR immunoreactive dots had β -arrestin counterparts was observed (Fig. 4e,f). These data demonstrate that β -arrestin and the β_2 AR are colocalized following agonist addition. The additional β -arrestin staining that did not localize with surface β_2 ARs probably reflects internal pools.

We next investigated whether β -arrestin is localized in clathrin-coated pits in agonist-treated cells. COS-1 cells transfected with β -arrestin and Flag-tagged β_2 AR were double-stained for β -arrestin and for either clathrin or AP-2. A distinctive punctate pattern of clathrin and AP-2 was observed, and this was not affected by the presence of isoprenaline (data not shown). Following agonist treatment, β -arrestin colocalized with both non-Golgi clathrin and AP-2 in plasma-membrane coated pits (Fig. 4g–j). Endogenous β -arrestin in RBL-2H3 cells transfected with the M1 muscarinic cholinergic receptor behaves in the same way, changing in an agonist-dependent manner from a diffuse to a punctate pattern coincident with clathrin and AP-2 (F.S., unpublished observation). Given the colocalization of surface β_2 AR, β -arrestin, and plasma-membrane clathrin and AP-2 after agonist challenge, we conclude that the β -arrestin/clathrin interaction occurs in intact cells in the presence of activated receptor.

An adaptor molecule linking cell-surface receptors destined for uptake with some coated-pit component was originally proposed to explain the receptor specificity of endocytosis¹⁴. AP-2 and AP-1, structural components of clathrin-coated pits that also promote clathrin assembly¹³, were termed adaptors on account of their

	% Clathrin binding (normalized)	% Cell surface receptor loss
β -arrestin	100 $\pm$ 3	40 $\pm$ 1
ABBA	33 $\pm$ 4	22 $\pm$ 4
AAAB	68 $\pm$ 9	17 $\pm$ 3
arrestin	6 $\pm$ 1	15 $\pm$ 3*

FIG. 3 Characterization of β -arrestin/arrestin chimaera binding to clathrin cages and effect on β_2 AR internalization. The general structures of the proteins are shown, with the β -arrestin portion in black and the arrestin portion in white. The proteins were translated *in vitro* and then analysed for direct binding to clathrin cages as described in Methods. Clathrin cage binding data were normalized to the binding for β -arrestin and represent the mean $\pm$ s.e.m. of at least three independent determinations. To characterize β_2 AR internalization, COS-1 cells, transfected with pBC- β_2 AR alone or co-transfected with a pBC-arrestin construct, were incubated with isoprenaline, washed, and then analysed for cell surface β_2 ARs as described in Methods. The data represent the mean $\pm$ s.e.m. from five independent experiments. Asterisk indicates that identical loss of cell-surface receptors was obtained with control (no arrestin co-transfection) and visual arrestin co-transfected cells.

interaction with the internalization motifs of several receptors^{15,16}, though the functional significance of these interactions is not entirely clear^{17,18}. An adaptor should recognize both receptor and clathrin lattice with high affinity, and these properties are fulfilled quite well by β -arrestin. However, unlike AP molecules, cytoplasmic β -arrestin apparently undergoes rapid recruitment from the cytoplasm to the plasma membrane in response to agonist activation and phosphorylation of the β_2 AR. Thus the β -arrestin-bound β_2 AR is targeted for internalization, and on encountering a coated pit¹⁹, β -arrestin may interact with the assembled clathrin lattice, resulting in selective endocytosis of the receptor. As β -arrestin has a lower affinity for clathrin than for the β_2 AR (ref. 12), we favour a model in which β -arrestin initially binds to the β_2 AR before interacting with clathrin. Although our results indicate that the agonist-activated β_2 AR is required to localize β -arrestin to clathrin-coated pits, potential β_2 AR/ β -arrestin interactions with other coat components and the mechanisms regulating β -arrestin/clathrin interaction remain to be elucidated. It seems likely that many other G-protein-coupled receptor signalling systems may

use a similar mechanism of arrestin-promoted internalization of agonist-activated phosphorylated receptors through clathrin-coated pits^{12,20,21}. □

Methods

Arrestin binding to clathrin cages and CVs. Bovine arrestin constructs were linearized, transcribed and translated using rabbit reticulocyte lysate and [³H]leucine²². The preparation of clathrin, clathrin cages and CVs²³, and the cage-binding assay²⁴ have been previously described. Clathrin cages (0.5 mg ml⁻¹) were crosslinked with 0.5 mM 3,3'-dithiobis(sulphosuccinimidyl) propionate (DSSP; Pierce) in 100 mM sodium-N-morpholino-ethanesulphonic acid (Na-MES), pH 6.5, 1 mM EGTA and 0.5 mM MgCl₂ for 30 min at 4 °C as described⁴¹. To prepare purified recombinant arrestins, β -arrestin and arrestin-3 complementary DNAs were subcloned into pTrcB (Invitrogen), transformed in BL21 cells, and induced with 30 μ M isopropyl- β -D-thiogalactoside (IPTG) for 17–20 h at 30 °C. The cell lysate was fractionated with ammonium sulphate, pelleted, resuspended, and sequentially applied to heparin- then Q-Sepharose.

To measure arrestin binding to clathrin cages, *in vitro* translated or purified arrestins were incubated in the presence or absence of clathrin cages in 50 μ l of 100 mM Na-MES, pH 6.8, 1 mM dithiothreitol, 1 μ g ml⁻¹ each of leupeptin, pepstatin and antipain (buffer A) for 20 min at 22 °C. Clathrin and bound proteins

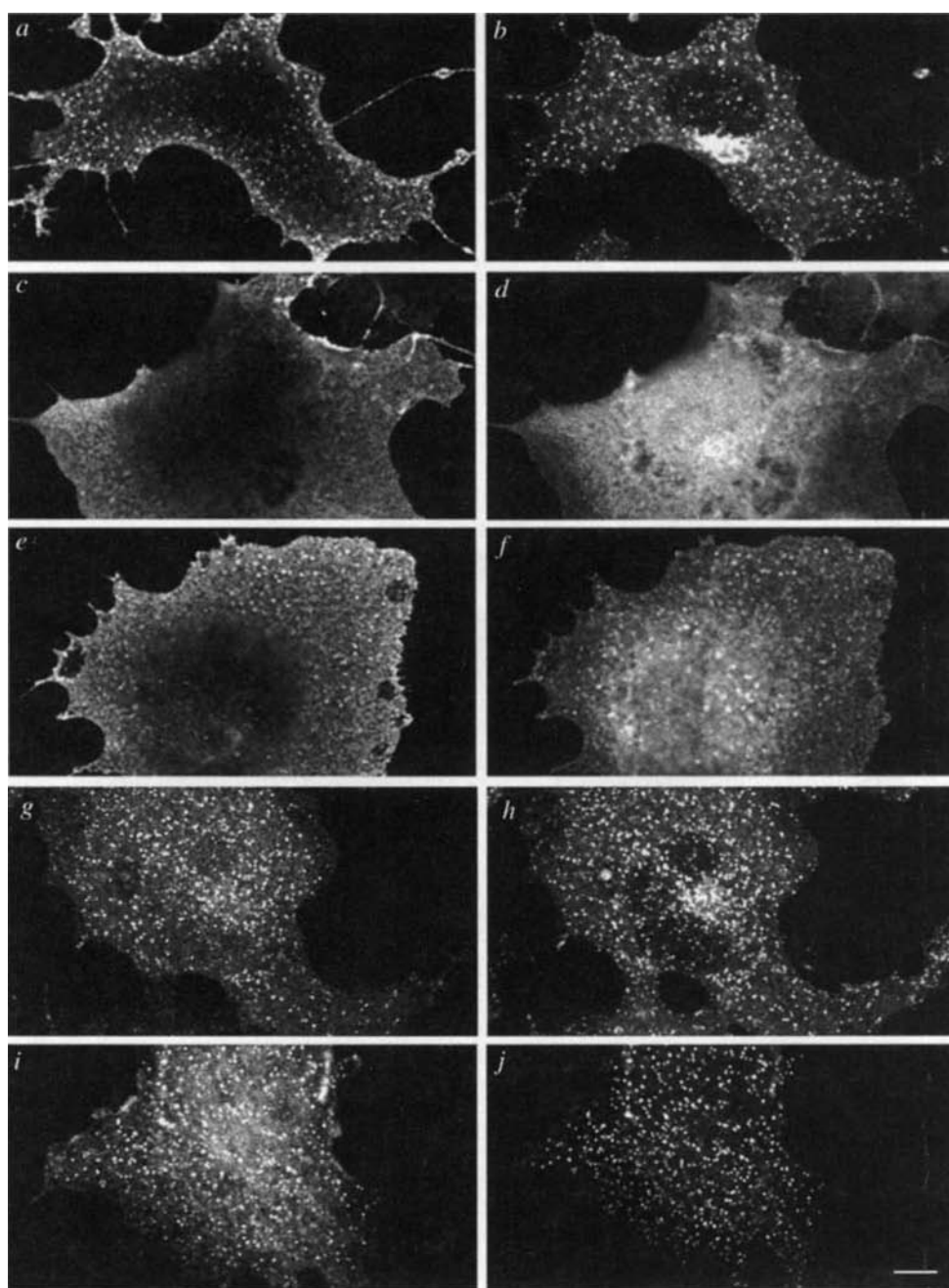


FIG. 4 Agonist-dependent colocalization of the surface β_2 AR and β -arrestin with clathrin-coated pits in COS-1 cells. Surface-localized β_2 ARs were visualized by immunofluorescence with anti-Flag antibodies after fixation but before permeabilization; β -arrestin, clathrin and AP-2 were localized in the same cells after detergent treatment, as described in Methods. β_2 AR (a) and clathrin (b) colocalization; β_2 AR (c, e) and β -arrestin (d, f) colocalization; β -arrestin (g, i) and clathrin (h) or AP-2 (j) colocalization. All cells were incubated with isoprenaline for 10 min except for those in c and d. Scale bar, 10 μ m.

were sedimented at 75,000 r.p.m. for 5 min at 4 °C in a TLA100 rotor. CVs and crosslinked cages (5 nM clathrin trimers) were incubated with purified arrestins in 50 µl of 120 mM potassium acetate, 20 mM HEPES buffer, pH 7.3, for 20 min at 22 °C and then centrifuged through a 100-µl 0.2 M sucrose cushion at 100,000 r.p.m. for 7 min at 4 °C. Pellets were resuspended in 20 µl SDS-sample buffer, heated at 100 °C for 5 min, and analysed by SDS-PAGE on a 7.5 or 10% gel. Subsequent analyses involved fluorography²², Coomassie-blue staining, or immunoblotting using the anti-arrestin monoclonal antibody F4C1.

Transfection and β_2 AR-internalization assays. Expression constructs were made by excising the open reading frames of bovine visual arrestin²⁵, β -arrestin²⁶, arrestin-3 (ref. 8), ABBA and AAB, blunting with Klenow, and then ligating into the expression vector pBC12BI. COS-1 cells were grown to ~80–90% confluence in T75 flasks at 37 °C in a humidified atmosphere containing 5% CO₂/95% air in Dulbecco's modified Eagle's medium (DM) supplemented with 10% fetal calf serum, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. Cells were transfected with 10–12 µg of pBC- β_2 AR, with or without 2–3.3 µg of the pBC-arrestin construct using 65 µl of lipofectAMINE following the manufacturer's instructions. The cells were trypsinized 48 h after transfection, washed twice with ice-cold PBS, and resuspended in PBS containing 0.1 mM ascorbic acid. Cells were incubated with or without 1 µM (–) isoprenaline at 37 °C for 15 min, washed 2–3 times with ice-cold PBS, and resuspended in 0.5 ml of PBS. Cell-surface receptors were measured by incubating with 10–15 nM [³H] CGP-12177 for 3 h at 14 °C in the presence or absence of 10 µM (–) alprenolol. Arrestin expression was assessed by immunoblotting using a 1:2,000 dilution of the monoclonal antibody F4C1 (ref. 27), which recognizes the epitope DGVVLVD, present in visual arrestin, β -arrestin, and arrestin-3. Blots were

washed, incubated with horseradish peroxidase-conjugated secondary antibody and visualized by chemiluminescence using ECL (Amersham).

Immunofluorescence microscopy. COS-1 cells were trypsinized 7 h after transfection, plated (5×10^4 cells per well) on 12-mm round glass coverslips in a 24-well plate with 0.5 ml medium per well, and analysed 24 h after transfection. The cells were incubated in HEPES-DMEM with or without 10 µM (–) isoprenaline for 10 min at 37 °C and processed for immunofluorescence analysis¹⁹. To detect surface Flag-tagged β_2 AR, cells were incubated with mouse monoclonal antibody M2 (10 µg ml⁻¹) (Eastman Kodak) in blocking buffer at 37 °C, washed, and fixed an additional 3 min with 3.7% formaldehyde. Total cellular clathrin, β -arrestin, or AP-2 were detected using rabbit polyclonal antiserum 27004 (1:100 dilution)²⁸ or mouse monoclonal antibody X22 (60 µg ml⁻¹) for clathrin, a rabbit affinity-purified antibody (3.2 µg ml⁻¹) raised against a C-terminal 16 amino-acid peptide for β -arrestin⁸, or mouse monoclonal antibody AP.6 (62 µg ml⁻¹) for AP-2, and appropriate secondary antibodies preadsorbed to minimize interspecies crossreactivity (Jackson ImmunoResearch). Confocal microscopy was performed on a BioRad MRC-600 laser scanning confocal microscope (Hemmelholsteadt, UK) attached to a Zeiss Axiocvert 100 microscope, using a Zeiss Plan-Apo 63 $\times$ 1.40 NA oil-immersion lens. Cells expressing the lowest levels of transfected proteins, but clearly above those of non-expressing counterparts in the same specimens, were chosen for view. Images of each fluorescent signal were collected sequentially in the photon-counting mode using single-line excitation. Specificity of labelling and absence of signal crossover were established by examination of single-labelled control samples.

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CORRESPONDENCE and requests for materials should be addressed to J.L.B. (e-mail: benovic@lac.jci.tju.edu).

Activation of specific RXR heterodimers by an antagonist of RXR homodimers

Deepak S. Lala*, Ranjan Mukherjee†, Ira G. Schulman‡§, Stacie S. Canan Koch||, Laura J. Dardashti||, Alex M. Nadzan||, Glenn E. Croston¶, Ronald M. Evans‡ & Richard A. Heyman*

Departments of * Retinoid Research, † Cardiovascular Research, || Retinoid Chemistry, ¶ New Leads, Ligand Pharmaceuticals, 10255 Science Center Drive, San Diego, California 92121, USA

‡ The Salk Institute for Biological Studies, La Jolla, California 92037, USA

RETINOID X receptor (RXR) plays a central role in the regulation of many intracellular receptor signalling pathways¹ and can mediate ligand-dependent transcription, acting as a homodimer or as a heterodimer^{1–6}. Here we identify an antagonist towards RXR homodimers which also functions as an agonist when RXR is paired as a heterodimer to specific partners, including peroxisome proliferator-activated receptor and retinoic acid receptor. This dimer-selective ligand confers differential interactions on

the transcription machinery: the antagonist promotes association with TAF110 (TATA-binding protein (TBP)-associated factor 110) and the co-repressor SMRT⁷, but not with TBP, and these properties are distinct from pure RXR agonists. This unique class of RXR ligands will provide a means to control distinct target genes at the level of transcription and allow the development of retinoids with a new pharmacological action.

Studies of the mechanisms by which intracellular receptors regulate transcription have been facilitated by synthetic ligands with different properties to naturally occurring hormones. For example, synthetic oestrogens and progestins with partial agonist/antagonist activities have helped to elucidate transactivating functions (AF-1 and AF-2) of the oestrogen receptor^{8,9}, leading to the development of new agents for hormone-replacement and antihormonal therapies. Synthetic retinoids have also enabled the specific contributions of heterodimer partners in transcriptional responses to be determined²³, receptor polarity and allosteric control of ligand binding to be examined^{22,10,11}, and transactivation to be separated from transrepression^{12–14}. The transcriptional responses elicited upon ligand binding to receptor are thought to be a consequence of conformational changes that promote interactions between receptor dimers, coactivators, co-repressors^{7,10,15} and the basal transcription machinery¹⁶.

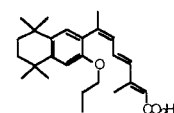
To study the molecular basis of RXR transcriptional regulation, we examined whether different RXR ligands could selectively modulate RXR-homodimer and RXR-heterodimer transcriptional activity. The ligand-binding properties of a series of retinoids (Fig. 1) were compared with their ability to transactivate members of the retinoic acid receptor (RAR) and RXR subfami-

§ Present address: Department of Retinoid Research, Ligand Pharmaceuticals, 10255 Science Center Drive, San Diego, California 92121, USA.

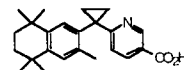
lies (Table 1). We identified two classes of synthetic compounds with unusual properties. Both LG100754 (ref. 17) and LG100268 (ref. 18) selectively bind to RXRs with nanomolar affinity, but each has only very weak affinity for the RARs ($>1,000$ nM; Table 1). In contrast, 9-*cis* retinoic acid (9-*cis* RA) binds with nanomolar affinity to both the RARs and RXRs^{19–21}. Although LG100754 binds RXR, it is unable to activate RXR homodimers (Table 1 and Fig. 2a). In contrast, LG100268 produces a concentration-dependent activation (half-maximal effective concentration, $EC_{50} = 4$ nM) that is consistent with its ligand-binding affinity (Fig. 2a). The ability of LG100754 to bind but not activate RXR indicated that it might function as an RXR antagonist. We examined the effect of LG100754 on transcriptional activation of an RXR homodimer by the RXR activator LGD1069 (ref. 22). LG100754 is a potent RXR antagonist, inhibiting transactivation in a concentration-dependent manner (half-maximal inhibitory concentration $IC_{50} = 20$ nM; Fig. 2b). It also antagonizes transactivation of RXR homodimers by LG100268 or 9-*cis* RA (Fig. 2c). Thus LG100754 is distinct from LG100268 in that it is transcriptionally neutral by itself and functions as a competitive RXR antagonist in the context of RXR homodimers.

We have shown that 9-*cis*-retinoic acid promotes a direct interaction between RXR and TBP as well as with a second component of the basal transcription machinery, TAF110 (ref. 16). Transcriptionally defective RXR mutants do not interact with TBP but still bind TAF110, indicating a functional relationship between ligand binding, interaction with TBP, and transcriptional regulation¹⁶. To examine the ligand-dependent interaction between RXR and components of the basal transcription machinery, we compared the ability of LG100268, LG100754 and 9-*cis* RA to modify the interaction of RXR with TBP and TAF110, by the yeast two-hybrid assay²³. 9-*cis* RA and LG100268, which transactivate RXR homodimers, promote a ligand-dependent interaction between RXR and TBP (Fig. 2d). In contrast, LG100754, which functions as an RXR antagonist, fails to promote interaction with TBP (Fig. 2d). Conversely, LG100754 promotes a strong association between RXR and TAF110 (>30 -fold), whereas 9-*cis* RA and LG100268 lead to a moderate increase (~ 5 -fold) (Fig. 2e). As there are quantitative and qualitative differences in RXR–TBP and RXR–TAF110 interactions in the presence of LG100754 and 9-*cis* RA/LG100268, these results indicate that individual ligands may produce different functional changes in the RXR carboxy-terminal transactivation domain (AF-2, τ c), leading to distinct protein–protein interactions. In this system, LG100268 and LG100754 therefore seem to direct RXR towards different constituents of the basal transcription machinery, although whether this happens in mammalian cells remains to be established.

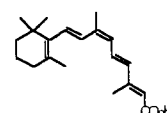
The activities of LG100754 and LG100268 were next compared in the context of RXR/peroxisome proliferator-activated receptor (PPAR) and RXR/RAR heterodimer pairs. RXR/PPAR α heterodimers are responsive to both RXR and PPAR ligands⁵. Thus, as expected, LG100268 activates the RXR/PPAR α heterodimer to produce a maximal induction of 4.5-fold at 1 μ M (Fig. 3a). Unexpectedly, LG100754 activates the heterodimer and is a stronger and more effective activator than LG100268, producing a 13-fold induction at 1 μ M. Thus LG100754 functions as both an antagonist of RXR homodimers and a transcriptionally active agonist of RXR/PPAR α heterodimers. Although ligands with mixed agonist/antagonist function have been reported for oestrogen receptors⁹, LG100754 is the first example of a mixed-function retinoid whose activity is dimer-selective.



LG100754
(2*E*,4*E*,6*Z*)-7-(3-*n*-propoxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)-3-methylocta-2,4,6-trienoic acid



LG100268
6-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-cyclopropyl]-nicotinic acid



9-*cis*-retinoic acid

FIG. 1 Structures and chemical names of LG100754, LG100268 and 9-*cis* retinoic acid.

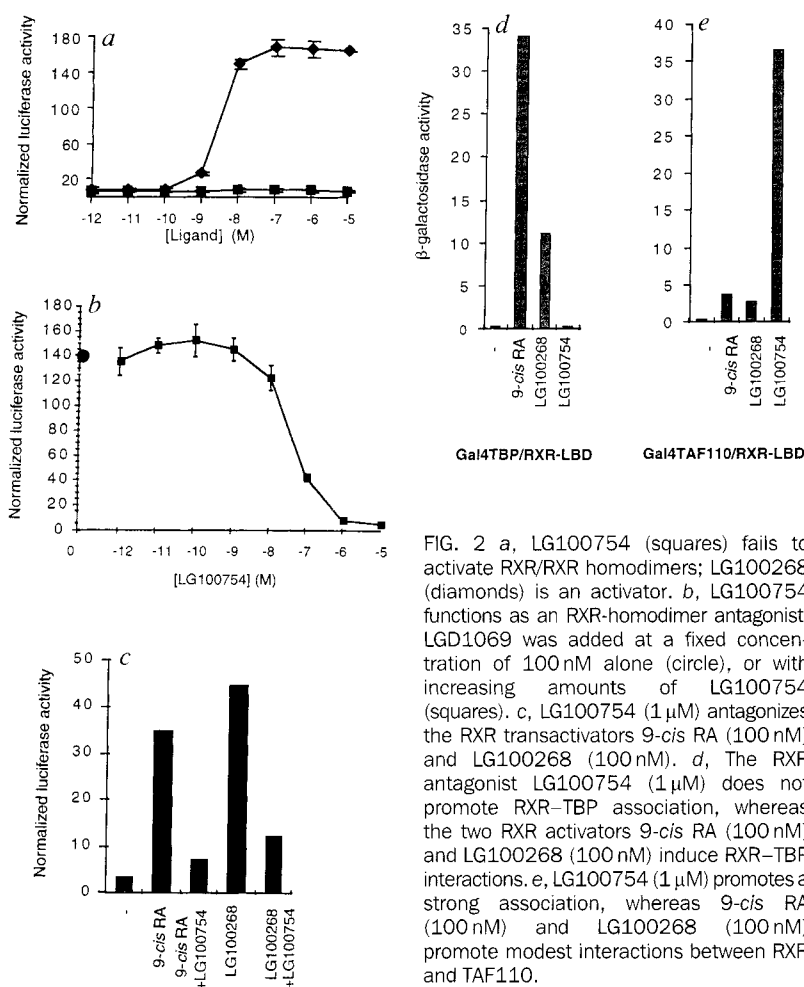


FIG. 2 a, LG100754 (squares) fails to activate RXR/RXR homodimers; LG100268 (diamonds) is an activator. b, LG100754 functions as an RXR-homodimer antagonist. LGD1069 was added at a fixed concentration of 100 nM alone (circle), or with increasing amounts of LG100754 (squares). c, LG100754 (1 μ M) antagonizes the RXR transactivators 9-*cis* RA (100 nM) and LG100268 (100 nM). d, The RXR antagonist LG100754 (1 μ M) does not promote RXR–TBP association, whereas the two RXR activators 9-*cis* RA (100 nM) and LG100268 (100 nM) induce RXR–TBP interactions. e, LG100754 (1 μ M) promotes a strong association, whereas 9-*cis* RA (100 nM) and LG100268 (100 nM) promote modest interactions between RXR and TAF110.

In contrast to PPAR, RAR suppresses RXR ligand binding and transactivation through allosteric interactions^{2,11}. However, when RAR is occupied, RXR ligands activate the heterodimer^{2,26}. To examine the effects of LG100268 and LG100754 on the transcriptional properties of the RXR/RAR heterodimer, we used co-transfection assays with: (1) wild-type RAR and RXR, together with a reporter (DR1, DR2 and DR5 response elements), and (2) a chimaeric receptor assay involving a Gal4-RXR ligand-binding domain (LBD) fusion protein^{2,27} and RAR-LBD along with a Gal4 reporter. Whereas RXR ligands, such as LG100268, by themselves do not activate the wild-type RXR/RAR heterodimer, LG100754 or the RAR-selective ligand TTNPB (ref. 22) strongly transactivates this heterodimer pair on the DR2 and DR5 reporter elements (Fig. 3*b* and *c*). Activation was not observed on the DR1 site, a classic RXR response element (data not shown). The addition of LG100754 and TTNPB together further enhances the transactivation (Fig. 3*b* and *c*), indicating that LG100754 is active on RXR/RAR heterodimers, and that either receptor within this dimer can be activated by its ligand while the partner remains unoccupied. Using the Gal4 heterodimers, we observe a response similar to wild-type RXR/RAR heterodimers; LG100754 produces a 50-

TABLE 1 Ligand-binding properties for RXRs and RARs

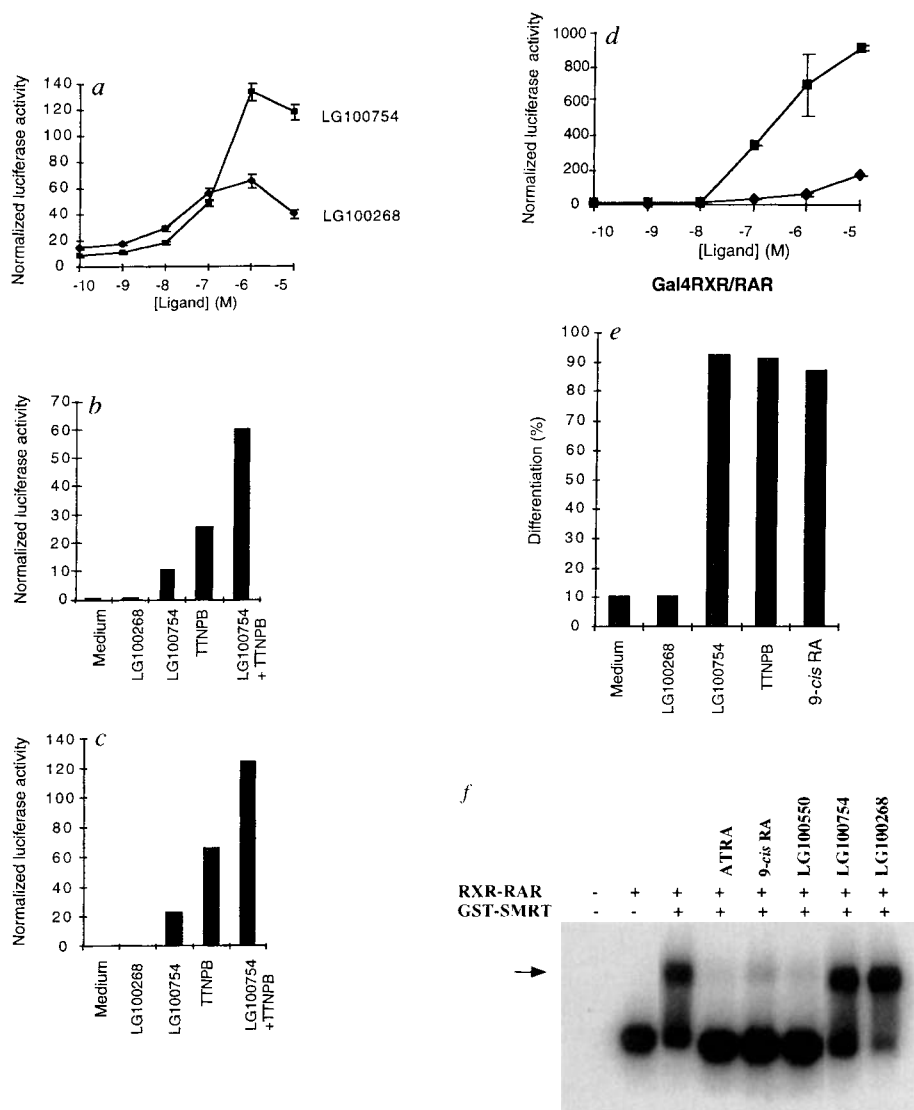
Compound	K_i values* (nM)					
	RAR- α	RAR- β	RAR- γ	RXR- α	RXR- β	RXR- γ
LG100754	>1,000	>1,000	>1,000	3.4	10	12.2
LG100268	>1,000	>1,000	>1,000	3.2	6.2	9.7
9- <i>cis</i> retinoic acid	15.2	13.4	14.7	6.7	6.2	9.7

The ligand-binding affinities for the various ligands for RXR and RAR were determined by their ability to displace [³H]9-*cis* retinoic acid or [³H]all-*trans* retinoic acid from RXR or RAR respectively.

* K_i values for the different ligands were determined using baculovirus-expressed receptors, and represent the mean of at least three separate determinations done in duplicate^{18,22}.

fold activation, whereas LG100268 is a very poor activator (Fig. 3*d*). These data indicate that certain RXR compounds escape suppression by RAR, and that the ability of LG100754 to activate RXR/RAR heterodimers is independent of the DNA-binding domain of the IR.

To determine whether these transcriptional responses occur in a cellular-based response, we studied the acute promyelocytic leukaemia (APL) cell line, NB4, which differentiates in response to retinoids such as 9-*cis* RA (ref. 28). LG100754, TTNPB and 9-*cis* RA, all of which activate RXR/RAR transcriptional pathways, induce differentiation (Fig. 3*e*), whereas LG100268, which cannot activate the RXR/RAR heterodimer, does not. This



implies that transactivation of RXR/RAR target genes is required for differentiation and that LG100754 can initiate the signalling pathways leading to this cellular response.

Transcriptional silencing of unliganded RAR is mediated through the corepressor SMRT (for silencing mediator for retinoid and thyroid-hormone receptors)⁷. Ligands binding to the RAR side of RXR/RAR heterodimers strongly dissociate SMRT, leading to activation. If LG100754 acts through RAR, it may also dissociate SMRT from RXR/RAR. We therefore used a gel-shift assay to investigate the ability of LG100754 to dissociate SMRT from RXR/RAR heterodimers. Ligands that bind directly to RAR (all-*trans* retinoic acid, LG100550 and 9-*cis* RA) dissociate SMRT (Fig. 3f). However, LG100268, which does not activate RXR/RAR heterodimers does not release SMRT from the complex. Likewise, LG100754 does not seem to dissociate SMRT from RXR/RAR heterodimers (Fig. 3f). Although preliminary data using the two-hybrid assay indicate some dissociation of SMRT by LG100754 (data not shown), this is still considerably weaker than RAR ligands. These results indicate that in the presence of SMRT, LG100754 and the RAR transactivators may differ in their interactions with RXR/RAR dimers. Although the exact mechanism of activation by LG100754 is unclear, each component of the receptor dimer may contribute to a mechanistically distinct pathway of activation.

We have identified the first RXR homodimer antagonist that also acts as an agonist on specific RXR heterodimers. The agonist activity of this ligand is not observed on all RXR heterodimer pairs, for example, RXR and the thyroid-hormone receptor (data not shown). RXR ligands initiate a selective cascade of interactions between receptor dimers, cofactors, and members of the basal transcription machinery, which may result from specific conformational changes within the RXR-LBD. These ligand-induced conformational changes could contribute to different transcriptional responses. The crystal structures of the RAR γ -LBD bound with all-*trans*-retinoic acid²⁹ and RXR α -apo-LBD (ref. 30) indicate that a 'mouse trap' mechanism may occur, whereby a ligand-induced conformational transition repositions the amphipathic α -helix of the AF-2-activating domain, forming a transcriptionally active receptor. Thus different ligands, upon entering the ligand pocket, may induce a repositioning of the α -helix to form active or inactive receptors. These synthetic compounds provide a new tool for dissecting RXR action and may yield pharmacological profiles with potential therapeutic value. □

Methods

Transfections. 5×10^4 cells were transfected with 100 ng receptor expression plasmid, 500 ng reporter and 500 ng β -galactosidase (β -gal) expression plasmid (internal control). Transfected cells were incubated overnight, washed and treated with charcoal-absorbed FBS medium $\pm$ retinoid. Cells were collected after 24 h and analysed for luciferase and β -galactosidase (β -gal) activity²⁴. To form RXR homodimers, pCMXR α was co-transfected into CV-1 cells with the reporter CRBP11-tk-LUC (ref. 6). To form RXR/PPAR α heterodimers, PPAR α was transfected into CV-1 cells with the reporter PPRE-tk-LUC as described²⁴. RXR/RAR activity on the DR2 element was examined using RXR and RAR expression plasmids and the reporter ApoA1-tk-Luc (ref. 25). RXR/RAR activity on a DR5 element was tested using a modified RXR (RX-PGR) (ref. 11) containing the P-box residues of the glucocorticoid receptor (GR) and a DR5 element containing a GRE (G5R) element to avoid basal activity from endogenous receptors¹¹. For the Gal4-RXR/RAR assay, CV-1 cells were co-transfected with pCMXGal4RXR α (ref. 2) and pCMXRAR α LBD (ref. 2). Concentrations of various ligands were fixed and are indicated in figure legends. For dose-response curves, compound concentrations are shown in the figures. Normalized response is the luciferase value divided by the β -galactosidase activity; all data points were performed in triplicate and varied by less than 20%. Where shown, error bars represent the standard deviation from the mean.

Yeast two-hybrid assays. Yeast strains and Gal4 DNA-binding-domain-TBP/TAF110 fusion-protein plasmids used to study RXR-TBP and RXR-TAF110 interactions have been described¹⁶. For β -gal assays, a minimum of three independent transformants were grown overnight at 30 °C, diluted, and ligands added¹⁶. Interaction between the proteins is indicated by increased β -gal activity. Ligand concentrations are indicated in the figure legends. Data points were performed in triplicate for each experiment and varied less than 20%.

NB4 cell-differentiation assays. These are described in ref. 28. Ligand concentrations are indicated in the figure legends. All points were performed in triplicate for each experiment and varied by less than 20%. Each experiment was repeated at least three times with similar results.

Band-shift assays. These are described in ref. 7. Ligand concentrations are indicated in figure legends.

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CORRESPONDENCE and requests for materials should be addressed to R.A.H. (e-mail: rheyman@ligand.com).

A new group of conserved coactivators that increase the specificity of AP-1 transcription factors

François-Xavier Claret*, Masahiko Hibi*†, Susheela Dhut‡, Takashi Toda‡ & Michael Karin*

* Department of Pharmacology, Program in Biomedical Sciences, School of Medicine, University of California, San Diego, La Jolla, California 92093-0636, USA

‡ Laboratory of Cell Regulation, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

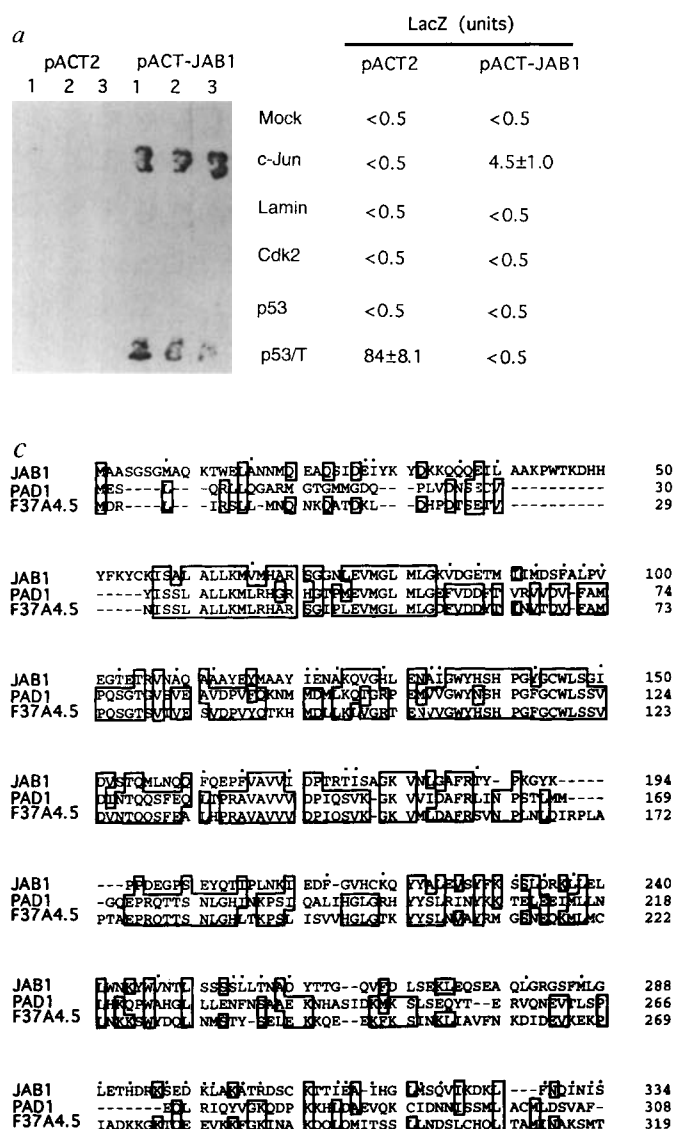
THE Jun proteins are nuclear proteins that combine with Fos proteins to form a gene-regulatory protein, AP-1. They have highly conserved DNA-binding and dimerization domains, resulting in almost identical sequence-recognition properties^{1–3}. Nevertheless, there are many indications that each Jun protein activates a distinct and only partially overlapping set of AP-1 target genes^{4–6}. Using the more variable activation domain of c-Jun as a bait, we identified a protein, JAB1, that interacts with c-Jun and JunD, but not with JunB or v-Jun. As a result, JAB1 selectively potentiates transactivation by only c-Jun or JunD. *In vitro*, JAB1 specifically stabilizes complexes of c-Jun or JunD with AP-1 sites and does not affect binding of either JunB or v-Jun.

† Present address: Department of Molecular Oncology, Biomedical Research Center, Osaka University Medical School 2-2, Yamada-Oka, Suita Osaka 565, Japan.

The amino-terminal half of JAB1 is very similar to the amino terminal region of Pad1 from fission yeast, which was identified genetically as a coactivator of a subset of AP-1 target genes⁷. JAB1 and Pad1 are also functionally interchangeable. They define a new group of coactivators that increase the specificity of target gene activation by AP-1 proteins.

A major problem in eukaryote gene regulation is achieving specificity in target-gene activation, given the large numbers of closely related activators with similar recognition properties. To reveal mechanisms that might further increase the specificity of target-gene activation by Jun proteins, all of which bind to AP-1 sites^{2,3}, we used a two-hybrid screen⁸ to identify proteins that selectively interact with c-Jun. The most variable domain of the Jun proteins is their N-terminal activation domain¹.

We used a portion of the c-Jun activation domain (cJ-AD) fused to the GAL4 DNA-binding domain (DBD) to screen a human B-lymphocyte complementary DNA library⁹. We identified two overlapping cDNAs that specifically potentiated activation of a GAL4-dependent *lacZ* reporter (Fig. 1a). An almost complete cDNA clone was isolated from a human T-lymphocyte library and its encoded protein was named JAB1 (for Jun-activation-domain-binding protein 1). Activity of constructs containing amino acids 1–223 or 43–223 of c-Jun was strongly potentiated by JAB1–GAL4AD expression in yeast, but deletion of amino acids 43–78 from cJ-AD abolished most of the response (Fig. 1b).



Although the basal activity of cJ-AD(1–79) was considerably lower than that of cJ-AD(1–223), it was still responsive to JAB1. Substitution of serines 63 and 73 with alanine did not affect this response, indicating it does not require c-Jun phosphorylation¹⁰. The chicken v-Jun AD was not responsive to JAB1 and had a considerably higher basal activity (Fig. 1b). These results suggest that amino acids 31–57 of c-Jun, which are absent in v-Jun¹¹, contain the JAB1-interaction surface.

Conceptual translation of the JAB1 sequence revealed an open reading frame encoding a polypeptide of relative molecular mass 37,500 (37.5K) (Fig. 1c), which was also detected after transfection of CV-1 cells with a JAB1 vector (data not shown). JAB1 is highly similar in its N-terminal region (57% identity) to the products of open reading frame F37A4.5 of *Caenorhabditis elegans*¹² and the *Saccharomyces pombe pad1+* gene⁷ (Fig. 1c). Interestingly, *pad1+* is necessary for target-gene activation by the *S. pombe* AP-1 transcription factor Pap1, a homologue of c-Jun⁷. The JAB1 transcript is 1.5 kilobases (kb) long and is widely expressed (data not shown). Immunofluorescence analysis indicated that JAB1 is a nuclear protein.

Transient transfections were used to assess whether JAB1 affects c-Jun-mediated transactivation in mammalian cells. Cotransfection of small amounts of c-Jun expression vector with increasing amounts of JAB1 into F9 (Fig. 2a) or HeLa (Fig. 2b) cells gave a dose-dependent potentiation of transactivation of the

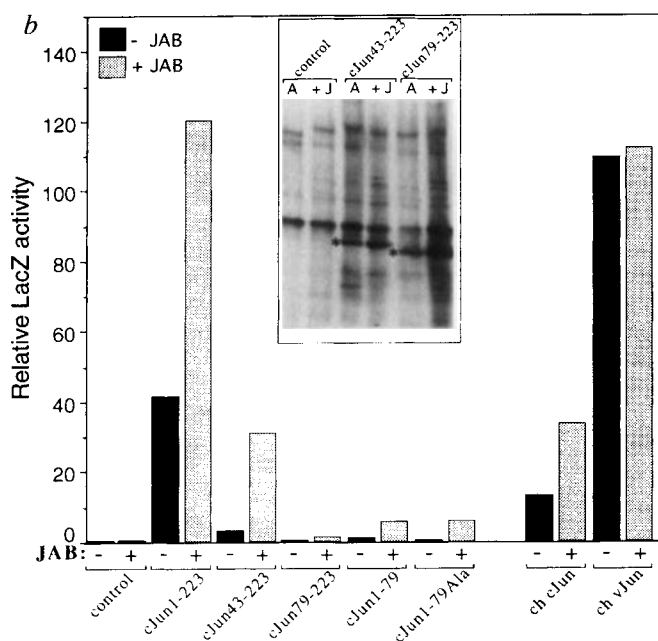


FIG. 1 Identification of JAB1. **a**, JAB1 specifically interacts with the c-Jun activation domain (cJ-AD) in yeast. pACT-GAL4AD(768–881) or pACT-JAB1-GAL4AD (clone 14) were transformed into *S. cerevisiae* cells together with plasmids encoding the GAL4 DNA-binding domain (DBD), either by itself (top, mock) or fused to: amino acids 1–79 of c-Jun, lamin, Cdk2 or p53. As a positive control, we coexpressed GAL4-DBD fused to p53 and GAL4-AD fused to SV40 large-T antigen (bottom). Filters containing transformants were stained for β -galactosidase expression (left panel). β -galactosidase activity determined by a solution assay is shown as average values ($\pm$ standard deviation) for three independent clones (right panel). **b**, Mapping the JAB1 interaction surface of c-Jun. The indicated portions of the human and chicken (ch) c-Jun and v-Jun ADs were fused to GAL4-DBD and tested for interaction with JAB1 by the two-hybrid system. The indicated values are averages of β -galactosidase activities determined on three independent clones. JAB1 does not affect the level of c-Jun–GAL4 expression in yeast, as detected by immunoblotting with GAL4-DBD antiserum (inset). A, c-Jun–GAL4-DBD; J, GAL4-DBD–JAB1. **c**, JAB1 is similar in sequence to Pad1 and F37A4.5. Boxes and dots indicate identical and similar amino acids, respectively. Broken lines represent gaps introduced to optimize the alignments.

AP-1-dependent -73Col-LUC reporter¹³. The potentiating effect of JAB1 was dependent on c-Jun expression and lessened as the dose of c-Jun expression vector was increased (Fig. 2a). Similar results were obtained using Pad1 expression vector (Fig. 2b). The effect was similar in magnitude to that of the previously identified coactivator CBP¹⁴. As JAB1 interacts with the cJ-AD in yeast, we examined its effect on transactivation by cJ-AD(1-223)-GAL4. Cotransfection with increasing amounts of either JAB1 or Pad1 resulted in dose-dependent potentiation of cJ-AD(1-223)-GAL4-mediated transactivation of 5 × GAL4-LUC reporter (Fig. 2c). This effect was not due to increased cJ-AD(1-223)-GAL4 expression (data not shown). JAB1 also potentiated activation by JunD-GAL4 but had no effect on activation by either JunB-GAL4 or v-Jun-GAL4 (Fig. 2d). Deletion of the first 43 amino acids of c-Jun did not affect the response to JAB1, but deletion of a further 13 amino acids abolished the response. These results were consistent with those of coprecipitation experiments demonstrating that a glutathione-S-transferase (GST)-JAB1 fusion protein can precipitate a GAL4 fusion protein containing the c-Jun and JunD but not the JunB or v-Jun activation domains from extracts of transiently transfected cells (Fig. 2e).

Transient transfections revealed that direct recruitment of JAB1 to the promoter, as a GAL4(DBD)-JAB1 fusion protein, does not result in transcriptional activation of the 5 × GAL4-LUC reporter, unless coexpressed with c-Jun (data not shown). Thus JAB1 lacks an autonomous activation domain. An alternative mode of coactivation may involve an effect on the binding of c-Jun to the AP-1 site TRE, the 12-*o*-tetradecanol phorbol acetate response element. We used electrophoretic mobility-shift assay (EMSA), under conditions in which only 10–20% of the collagenase TRE probe is bound by c-Jun, to examine the effect of JAB1 on DNA binding. GST-JAB1 was found to enhance binding of c-Jun to the TRE fourfold (Fig. 3a). EMSA using

other Jun proteins indicated that JAB1 also enhanced binding of JunD to the TRE, but had no effect on the binding of JunB or v-Jun (Fig. 3b).

No substantial effect of JAB1 was found on the rate of association of c-Jun with the TRE (Fig. 3c, top). But when the c-Jun-TRE complex was formed and then challenged with excess cold TRE oligonucleotide, we found that the presence of JAB1 resulted in considerable stabilization (fourfold) of the complex (Fig. 3c, middle and bottom panels). Similar results were obtained when the effect of JAB1 on the binding of GAL4(DBD)-Jun chimaeras to the GAL4 binding site was measured (data not shown). By and large, there was good correlation between the ability of JAB1 to interact with a given Jun protein, enhance its transactivating ability, and stabilize its binding to the TRE.

As the yeast Pad1 protein can potentiate c-Jun-dependent AP-1 transcriptional activity in mammalian cells (Fig. 2b), we tested whether JAB1 behaves similarly to Pad1 in *S. pombe*. Three functions have been attributed to *pad1*⁺: coactivation of the Pap1 target gene *p25*, resistance to caffeine and staurosporine, and maintenance of higher-order chromosome structure^{7,15}. The drug-resistance phenotype is due to potentiation of Pap1 transcriptional activity^{7,16}. The results shown in Fig. 4a indicate that expression of JAB1 from a high-copy-number vector in wild-type *S. pombe* cells was as efficient as *pad1*⁺ or *pad1*⁺ overexpression in conferring drug resistance. This effect was *pad1*⁺ dependent, as it was not observed in a *pad1*-deficient strain. In addition, expression of JAB1 in wild-type but not in *pad1*⁺-deficient cells induced p25 expression (Fig. 4b). No effect of JAB1 on Pap1 expression was observed. So far, however, we have not been able to assess whether JAB1 can complement a *pad1*-deficient strain owing either to technical complications or to the inability of JAB1 to assume certain essential functions of *pad1*⁺. These functions are not related to potentiation of Pap1 transcriptional activity because deletion of *pad1*⁻ is not lethal, whereas *pad1* deletion is⁷. To our

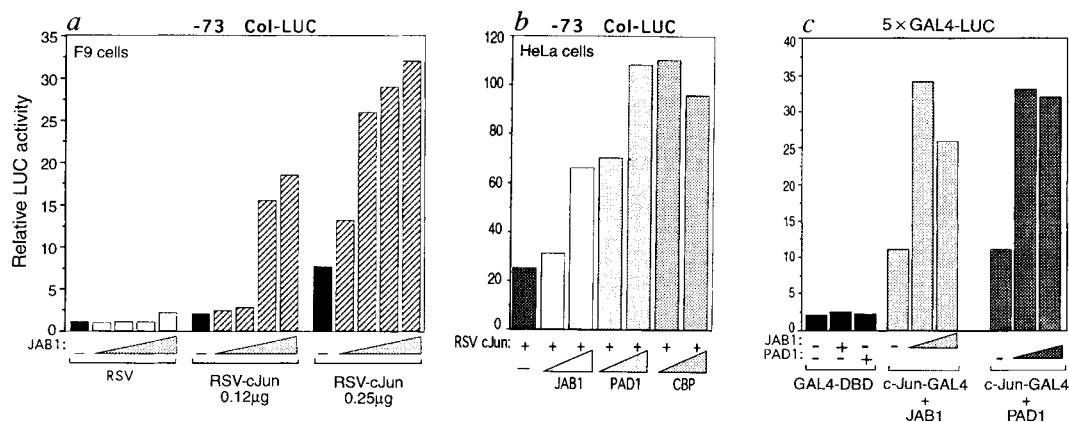
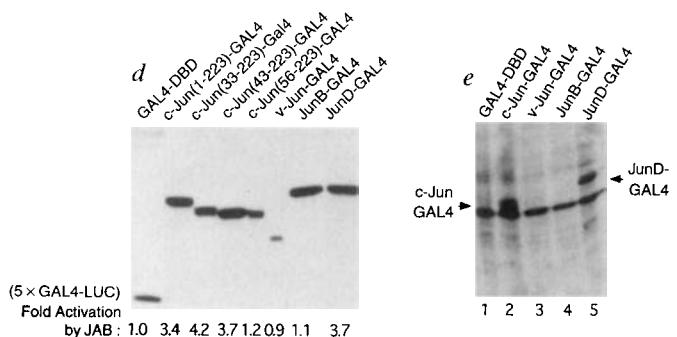


FIG. 2 JAB1 interacts with c-Jun and stimulates its activity in mammalian cells. **a**, JAB1 potentiates activation of the collagenase promoter. The -73 Col-LUC reporter (1 μ g) was cotransfected into F9 cells with various amounts of pCMV-JAB1 (0.25, 0.5, 1 and 2 μ g DNA) and either a c-Jun expression vector or an empty expression vector. Results are expressed as relative luciferase (LUC) activity. Expression of the -60 Col-LUC reporter lacking an AP-1 binding site¹³, was not affected (data not shown). **b**, JAB1 and Pad1 stimulate c-Jun-dependent transactivation. HeLa cells were transiently transfected with either 50 or 400 ng of pCMV-JAB1, -Pad1 or -CBP in the presence of RSV-c-Jun (200 ng) and -73 Col-LUC reporter (1 μ g). **c**, JAB1, Pad1 potentiate transactivation by cJ-AD(1-223)-GAL4. CV-1 cells were transiently cotransfected with the 5 × GAL4-LUC reporter (1 μ g) and expression vectors (0.1 μ g each) encoding either GAL4-DBD(1-147) or cJ-AD(1-223)-GAL4 and either 0.5 or 1.0 μ g of either pCMV-JAB1 or pCMV-Pad1 and a matching amount of pCMV. **d**, JAB1 potentiates activation by c-Jun and JunD. Top, immunoblot with GAL4-DBD antiserum of various Jun-GAL4-DBD expressed proteins. Bottom, effect of JAB1 on transactivation of the 5 × GAL4-LUC reporter in CV-1 cells by the various Jun-GAL4-DBD fusion proteins. **e**, Interaction of Jun-GAL4 fusion proteins



(expressed in CV-1 cells) with JAB1. JAB1-associated proteins were eluted and separated by SDS-PAGE and immunoblotted with GAL4-DBD antibodies.

knowledge, JAB1 is the first mammalian coactivator that enhances expression of endogenous genes in yeast.

As JAB1 and Pad1 are functionally interchangeable, they define a new family of eukaryotic coactivators that potentiate activation by certain members of the AP-1 family. Through selective interaction with the Jun proteins, JAB1 can increase the specificity of target-gene activation by this large family of related transcription factors. Although the detailed biological functions of JAB1 in mammalian cells have not been identified, the remarkable functional conservation between JAB1 and Pad1, especially the ability of JAB1 to confer drug resistance in yeast cells, suggests that it may regulate drug-resistance genes. In this respect it is notable that expression of the human multidrug-resistance *MDR1* gene is stimulated by Ras¹⁷, alkylating agents or ultraviolet irradiation¹⁸, all well-known stimuli of c-Jun and AP-1 activity^{19,20}. Although, JAB1 does not form a heterodimer with c-Jun or JunD, its effect on their DNA binding and transactivation abilities is analogous to the effect of the Extradenticle coactivator on homeodomain proteins of the Bithorax complex^{21,22}. Similarly, expression of JAB1 is likely to favour the activation of c-Jun and JunD target genes over those regulated by JunB. Additional specificity is contributed by the differential phosphorylation of the Jun proteins (T. Kallunki *et al.*, manuscript in preparation) which affects their interaction with phosphorylation-dependent coactivators such as CBP¹⁴. □

Methods

Two-hybrid system. Portions of cJ-AD were fused to GAL4-DBD(1–147) using the pGBT9 vector²³ and completely sequenced. They were tested for activation of the GAL4-dependent *lacZ* reporter²³ and cJ-AD(1–79)-GAL4 was chosen for library screening, because of its low basal activity and ability to activate transcription in mammalian cells²⁴. Human lymphocyte cDNA library (gift from S. Elledge) was used for screening, as described⁹. Two positives (c11, c14) were obtained out of two million transformants. The complete coding region was isolated from a λ gt11 Jurkat cDNA library (gift from G. Crabtree) and used to construct a JAB1-GAL4-AD expression vector by standard procedures. Filter assays of β -galactosidase activity have been described²⁵. 3-ml yeast cultures were inoculated into SC medium lacking tryptophan, leucine and uracil. Half of each culture was used to determine β -galactosidase activity²⁵ and a portion of the other half was lysed in SDS sample buffer and separated by SDS-PAGE. Proteins were electroblotted onto nitrocellulose and analysed using the Amersham ECL western blotting system with GAL4-DBD antiserum (gift from Y. Shaul). The complete nucleotide sequence of JAB1 was determined on both strands (Genbank accession number U65928).

Assay of transient expression and protein-protein interaction. F9, HeLa and CV-1 cells were cultured and transfected as described^{26,27}. The CMV-Pad1 expression vector was constructed by standard procedures (strategy available upon request); the CBP expression vector has been described²⁴. v-Jun(1–144)-GAL4, JunB(1–259)-GAL4 and JunD(1–256)-GAL4 expression vectors were constructed in the same way as c-Jun-GAL4 fusions^{19,28}. JAB (full-length, residues 1–333) was cloned into pGEX3X and the expressed protein purified as described¹⁹. For GST pull-down experiments, cells were lysed in HNT 0.4 M buffer (25 mM HEPES, pH 7.7, 0.4 M NaCl, 1.5 mM MgCl₂, 2 mM EDTA, 0.5% Triton X-100, 3 mM DTT, 1 μ g ml⁻¹ each of leupeptin, pepstatin, benzamide,

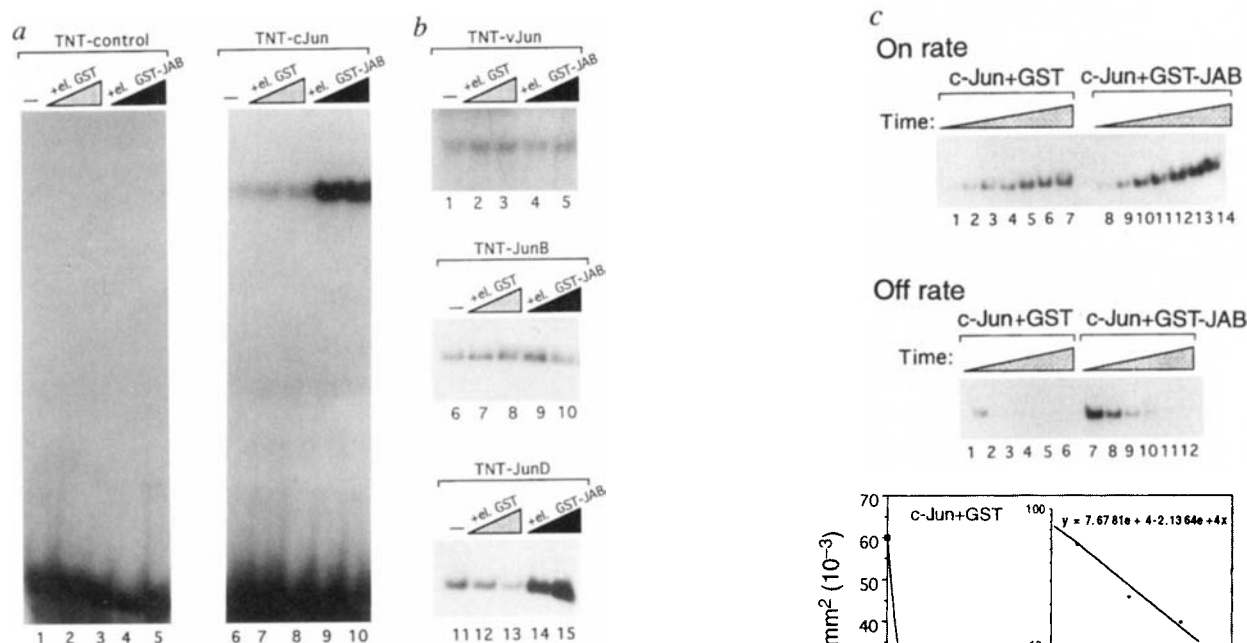


FIG. 3 JAB1 stimulates the binding of c-Jun and JunD to the AP-1 site. *a*, EMSAs were performed either with non-programmed or c-Jun-programmed reticulocyte lysates, incubated in the absence or presence of increasing amounts of either GST or GST-JAB1 proteins (400 and 800 ng each), and a collagenase TRE probe, as indicated. *b*, Same as *a*, except that lysates containing cell-free-translated v-Jun, JunB and JunD were used. *c*, JAB1 stabilizes the binding of c-Jun to the TRE. To determine the on-rate of c-Jun binding to the TRE probe, samples were removed at 0, 1, 2, 4, 8, 12 and 16 min and analysed for DNA binding. For the off-rate, the c-Jun-TRE complex was formed as described, and after 10 min, a 50-fold molar excess of unlabelled TRE oligonucleotide was added and samples were removed at 0, 2, 4, 8, 16 and 30 min. Quantified results from phosphorimaging are shown (bottom panels).

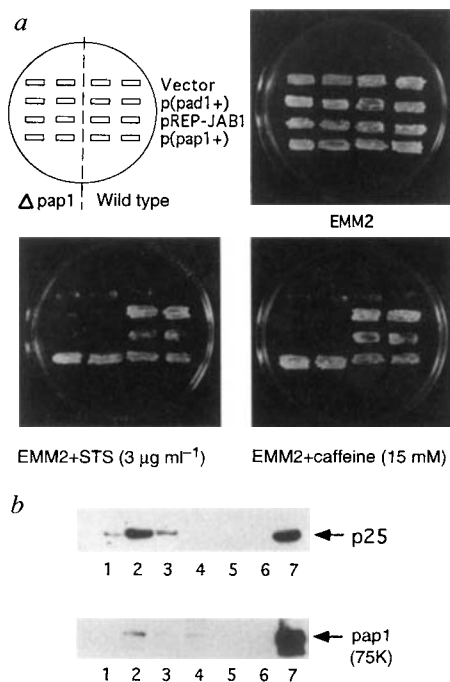


FIG. 4 JAB1 potentiates AP-1-dependent transcription in fission yeast. *a*, JAB1 confers drug resistance in yeast. Wild-type (HM123) or $\Delta pap1$ (TP108-3C) *S. pombe* strains⁷ were transformed as indicated and drug-resistant phenotypes examined. Two independent transformants for each plasmid were streaked on a minimal plate EMM2²⁹ (top right) or on minimal plates containing 3 µg ml⁻¹ staurosporine (STS, bottom left) or 15 mM caffeine (bottom right) and incubated for 4 d at 30 °C. Plasmids used were the empty expression vector pREP1, a *pad1*⁻-containing plasmid (pST23-6)⁷, a JAB1 expression vector (pREP41-JAB) and *pap1*⁻-containing plasmids (pST12 for wild type and pST22 for $\Delta pap1$)¹⁵. *b*, The transformants used in *a* were grown in minimal medium and cell extracts prepared. Pap-1-dependent transcription and protein expression were examined by immunoblotting with anti-p25 (upper) and with Pap-1 antiserum (lower). Transformants: wild type containing either the pREP1 vector (lane 1), a *pad1*⁻ plasmid (lane 2), JAB1 expression vector (lane 3), or $\Delta pap1$ containing a pREP1 (lane 4), a *pad1*⁻ plasmid (lane 5) or a JAB1 expression vector (lane 6), or a *pap1*⁻ plasmid (lane 7).

aprotinin and antipain). Lysates were precleared by incubation with GST-coated glutathione-agarose beads. The beads were spun down and the supernatants diluted fourfold with HNT 0 M (without NaCl) buffer before incubation with GSH-agarose beads coated with 4 µg purified GST-c-Jun(1-223), GST-JAB1 or GST¹⁹ for 2 h at 4 °C. Beads were spun down and washed 4 times with HNT 0.1 M buffer, resuspended in SDS-sample buffer and analysed by SDS-PAGE, immunoblotting with GAL4-DBD antiserum and visualized by ECL system.

Electrophoretic mobility-shift assays. Plasmids encoding c-Jun, v-Jun, JunB and JunD cDNAs^{19,28} were used for coupled *in vitro* transcription-translation reactions using the TNT reticulocyte-lysate system (Promega). Expression of different proteins was assessed by ³⁵S-methionine labelling and SDS-PAGE (data not shown). Lysates containing similar amounts of either c-Jun, v-Jun, JunD or JunB or unprogrammed lysates (0.5 µl each) were incubated with either purified GST or GST-JAB1 (400 and 800 ng of each protein) and 3 µg poly(dI-dC) on ice for 15 min in 20 µl containing 12% glycerol, 12 mM HEPES (pH 7.9), 60 mM KCl, 5 mM MgCl₂, 4 mM Tris-HCl (pH 7.9), 0.6 mM EDTA and 5 mM DTT. A ³²P-labelled double-stranded-oligonucleotide collagenase TRE probe¹³ was added to the binding reaction (10,000 c.p.m.) and incubated for a further 15 min at room temperature. Protein-DNA complexes were separated on a 5% native polyacrylamide gel.

***S. pombe* strains.** 972(h⁻), HM123(h⁻leu1), TP108-3c(h⁻leu1 *ura4 pap1::ura4*⁺) and TP42(h⁻/h⁺ leu1/leu1 *ura4/ura4 his2+ade6-M210/ade6-M216 pad1::ura4+/-*). Complete medium, YPD, modified synthetic EMM2, and malt-extract medium for mating and sporulation were prepared as described²⁹. Plates contained 1.6% agar. Standard procedures for *S. pombe* were followed²⁹; *S. pombe* cells were transformed using the lithium method³⁰ and extracts prepared by disrupting the cells with glass beads.

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CORRESPONDENCE and requests for materials should be addressed to M.K. (e-mail: paiford@ucsd.edu).

ERRATUM

Complex burrows of the mud shrimp *Callinassa truncata* and their geochemical impact in the sea bed

W. Ziebis, S. Forster, M. Huettel & B. B. Jørgensen

Nature **382**, 619-622 (1996)

THE e-mail address of the corresponding author (W.Z.) was incorrect. It is: wiebke@postgate.mpi-mm.uni-bremen.de □

CORRECTION

Requirement of mammalian DNA polymerase-β in base-excision repair

Robert W. Sobol, Julie K. Horton, Ralf Kühn, Hua Gu, Rakesh K. Singhal, Rajendra Prasad, Klaus Rajewsky & Samuel H. Wilson

Nature **379**, 183-186 (1996)

THE second sentence of the main text should read: "When bred, these mice fail to produce offspring that are homozygous for the β-pol deletion mutation, and embryos harbouring the homozygous deletion mutation are present at day 10.5 of gestation in the expected mendelian ratios". □

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Molecular medley

Techniques and technology for the molecular biologist — prepour, polyacrylamide gels for DNA sequencing, a universal support for DNA synthesis, a baculovirus expression system, a telomerase detection kit and cDNA synthesis kits.

AFTER much research and development, Stratagene has released its first DNA sequencing system featuring **prepoured, polyacrylamide gels**. The CastAway precast sequencing gels eliminate the need to manually pour sequencing gels, significantly reducing the time involved in gel preparation. Traditional methods can take up to 2 h of preparation to clean the plates, mix the solutions, degas the acrylamide, pour the plates and wait for polymerization. The company states that CastAway gels are ready to run in less than 5 min. The procedure requires the user simply open the bag, place the gel in the sequencing device, run the gel and dry in the gel dryer. These precast gels are thinner than standard gels, resulting in shorter run times and reduced band compressions. And, there is no need for filter paper and less risk of gel tearing because the precast gels are covalently bound to one of the glass plates. The vertical sequencing device, exclusively designed for use with the precast gels, is simple and safe to operate — gels snap in and are ready to run. The CastAway system also includes convenient, stackable fixing trays and a radioactive storage container.

Reader Enquiry No. 100

Quantiplex **branched-DNA assays** from Chiron Diagnostics have been formulated to provide direct measurement of viral nucleic acid levels in patients with HIV, hepatitis C (HCV) and B virus and cytomegalovirus. Quantification of viral DNA



Quantiplex for viral nucleic acid levels.

and RNA in clinical samples is becoming increasingly significant in the management of viral infections. Measurement of the level of HCV RNA is considered particularly useful as only some 25 per cent of HCV-infected individuals respond to inter-

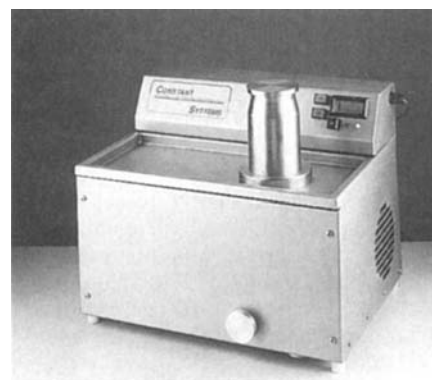
feron- α 2b, the approved treatment, and relapse is common. Studies using bDNA to quantify HCV RNA indicate that there is a strong correlation between the level of RNA before therapy and the subsequent response to treatment; the higher the pretreatment level of RNA, the less promising the long-term response. These assays should enable clinicians to predict who may or may not respond to standard therapeutic regimes and allow them to target those unlikely to respond to more aggressive treatment. In the case of HIV, long-term survivors have generally been shown to have lower viral load than those who progress more rapidly to AIDS, thus it may be preferable to include patients in anti-viral treatment trials according to their viral load rather than CD4 levels.

Reader Enquiry No. 101

BioGenex offers **CPG's universal support** that is designed to be used with all DNA syntheses irrespective of the first (3') base in the sequence. There is no modification to the standard synthesis protocols and only a minor modification of the deprotection protocols (only the introduction of 1–2 per cent lithium chloride is required). Deprotection and cleavage of the oligonucleotide can be done utilizing fast or normal synthesis chemistries. The new support contains an adaptor attached to CPG and chain elongation with the first base in the sequence attached to the adaptor. Moreover, the synthesis can be performed either 'trityl on' or 'trityl off'. During the cleaving and deprotection step the adaptor degrades, leaving the synthesized oligonucleotide free in solution. This cleavage is complete and produces an oligonucleotide that does not include any chemical contribution from the support. The CPG universal support is available in bulk or prepacked in synthesis columns for most commercial synthesizers in 40-nmol, 0.2- μ mol and 1.0- μ mol sizes.

Reader Enquiry No. 102

The new One Shot **cell disrupter** machine from Constant Systems handles up to 10 ml per shot of bacterial, fungal, yeast, mammalian, plant or algal cells. This includes filamentous cells and solid samples, such as seaweed, mammalian and plant tissues. Moreover, this system can be used for general disruption, including the release of subcellular and intracellular particles, as well as nucleic acids and pro-



Constant Systems' cell disrupter system.

teins. The principle of disruption this system is the use of high pressure to force a sample through a small orifice at high speed. Pressure is adjustable up to 2.8×10^5 KPa and stable and consistent during the disruption cycle. The disruptor can complete a cycle in less than 30 s, has a clean-up time of about 2 min and does not require any consumable parts.

Reader Enquiry No. 103

DNA cloning

Life Technologies now offers **thermo-sensitive alkaline phosphatase (TsAP)**, a phosphomonoesterase purified from *Escherichia coli* expressing a modified bacterial alkaline phosphatase gene. This enzyme hydrolyses 5'-phosphate groups from DNA, RNA and nucleotides. TsAP has higher specific activity than wild-type bacterial alkaline phosphatase and can be used at 37 °C and 65 °C for dephosphorylating recessed and blunt DNA ends. It can be heat-inactivated in the presence of EDTA, allowing users to proceed directly into a ligation reaction without having to do phenol extractions or ethanol precipitations. Incubation time is said to be reduced to 15 min, decreasing overall reaction time.

Reader Enquiry No. 104

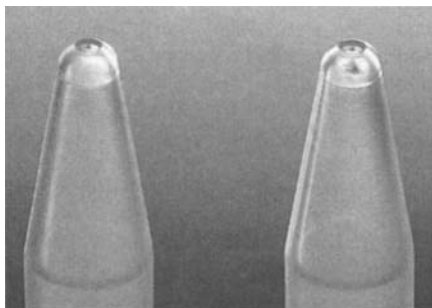
Cambio now offers Epicentre Technologies' **Fast-Link DNA ligation and screening kit**. Specifically developed to increase the speed and efficiency of the DNA cloning procedure, the kit allows ligation of inserts containing overhangs or blunt ends to be performed in about 5 min, as well as the ligation of PCR products with A overhangs into T vectors, which can be per-

formed within an hour. Following ligation and transformation, the gel lysis technique can be used for recombinant screening, allowing the rapid screening of colonies for insert-containing plasmids. By using DNA ligation, transformation and recombinant screening can be done in as short as 24 h, dispensing with the need to grow cultures and perform mini-preps.

Reader Enquiry No. 105

PCR

SeeDNA, a new co-precipitant from Amersham, has been engineered to overcome some of the problems associated with ethanol precipitation of small quantities of nucleic acid. The co-precipitant allows precipitated material to be seen as vivid pink pellet, clearly visible under normal conditions, or as a fluorescent pellet under ultraviolet light. The 5-min protocol facilitates the recovery of even minute quantities (less than 2 ng ml⁻¹) of nucleic acid and forms tight pellets, which do not spread along tube walls. Once precipitated, the nucleic acid can be readily re-



Visualize precipitated DNA more easily with SeeDNA from Amersham.

solubilized as SeeDNA dissolves along with the sample. The co-precipitant consists of a brightly coloured, polymer-based carrier molecule that is chemically inert and does not interfere with subsequent experimental procedures. It has been shown to be compatible with many applications including sequencing, PCR, transformations, cDNA, synthesis, and

in vitro transcription and translation, according to the manufacturer. Because the fluorophore absorbs in the ultraviolet region, it is not recommended for use in procedures that require spectrophotometric measurements or methods that are based on rhodamine detection.

Reader Enquiry No. 106

The application of Igen's Origen electrochemiluminescent technology to the quantitative measurement of PCR has led to the development of assays that are said to be simple, rapid, sensitive and precise. These assays have been developed with more than three logs of dynamic range for the measurement of DNA and RNA sequences. The system has been shown to quantify sequences between 30 and 1,000 base pairs using no wash formats, although this sample range can probably be extended. Quantification has been made down to two target DNA copies in this size range. Moreover, the sensitivity is said to allow for a reduction in PCR cycles, thereby increasing assay precision and reproducibility. There are no known hybridization buffers that interfere with detection, and the sequences can be measured in 5 mg ml⁻¹ of matrix protein.

Reader Enquiry No. 107

Sanyo Gallenkamp is pleased to announce the launch of their new **PCR thermal cycler**, to be used for the patented polymerase chain reaction (PCR) process. This is a thermal cycler that offers rapid heating and cooling to ensure successful DNA amplification. The MIR-D30 achieves this performance by using the unique dynamic heat block exchanger. Rapid heating and cooling are achieved by the placing a hot or cool block underneath the sample block. The use of microprocessor PID and feed-forward control allows the heating/cooling block to move into position and back again at precisely the right time. This minimizes the risk of overshoot, especially important at higher temperatures. Using two separate blocks for heating and cooling prevents the thermal stresses that are imposed on combined heating/cooling blocks.

Reader Enquiry No. 108

Biometra has introduced its new generation of **thermocyclers**, UnoII. These thermocyclers possesses an interchangeable block module, which can be replaced easily. A choice of block modules for 0.5-ml tubes (48-format), 0.2-ml tubes (96-well format), microscope slides (four-position) or even a block for a 384-well microtitre plates. The heart of these units is a newly devel-



BioMetra's UNOII thermal cycler uses a gold-plated silver thermal-cycling block for improved heat conduction.

oped block made of silver and coated with a gold layer to prevent corrosion. Using silver improves the high heat conductivity, with maximum heating of >3.0 °C s⁻¹ and cooling of 2 °C s⁻¹. The long-life Peltier-elements are microprocessor controlled every 50 ms to ensure optimal block uniformity and reproducible cycling conditions. The heated lid can now be adjusted to the height of many reaction vessels, including tubes or plates. When lowering the heated lid an automatic clinch protects the vessel against damage. The interchangeable blocks should allow the user to buy a standard instrument, but use the instrument later for *in situ* applications or high-throughput screening with the 384-well plate.

Reader Enquiry No. 109

Purification

Schleicher & Schuell now offer a wide selection of products for **isolation, purification and concentration of DNA and RNA** based on a variety of separation technologies. The Elu-Quik DNA purification kit has been engineered for the isolation of genomic DNA from a variety of sources, including plasmid mini preps and cleaning procedures. It is said to be suitable for DNA ranging from 500 base pair to 200 kilobases. Elutip mini-columns are intended for the purification of oligonucleotides after synthesis, cleaning procedures, labelling reactions and PCR procedures. They are designed for gentle elimination of particles and containments that may cause high background levels or interfere with sample activity. IsoCode Stix is a new blood collection matrix, which is designed to provide fast and efficient isolation of DNA-template for PCR from whole blood within a stated 35 min. It also simplifies storage and archiving of blood samples from almost any source such as transgenic animals or clinical research samples, and are adaptable for automation. Also available is an NA 45 DEAE cellulose membrane for isolation of DNA fragments from agarose gels and is recommended for the purification of

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READER ENQUIRY NO. 68

PCR-products. The Biotrap Series is an electroelution device for large-scale isolation and purification of most kinds of nucleic acids ranging from 10-base nucleotides to 150 kb.

Reader Enquiry No. 110

Ambion has introduced the Poly(A) Pure and Micro Poly(A) Pure kits for the **rapid purification of mRNA directly from tissue or cells** without the need for total RNA isolation. Both kits use oligo-d(T) cellulose, which has been shown to have a high binding capacity for mRNA. Yields of Poly(A)⁺ RNA from 1 g of tissue are in the stated range of 60 µg. The Poly(A) Pure kit contains enough reagents for seven isolations from samples of 0.2–1.0 g tissue or 0.2–2.0 mg of total RNA. The Micro Poly (A) kit contains enough reagents for 20 isolations from samples of 10–220 mg of tissue or 2–400 µg of total RNA.

Reader Enquiry No. 111

Molecular means

Epicentre's Plasmid-Safe DNase has been designed as a fast, easy method to selectively remove contaminating bacterial chromosomal DNA remaining in preparations of plasmid, COSMID or BAC DNA. This DNase digests linear dsDNA, closed-circular and linear ssDNA, but has no activity on nicked or closed circular dsDNA. Following digestion, the enzyme is inactivated simply by incubation at 70 °C for 30 min. Treatment with Plasmid-Safe DNase as a final purification step is stated to prevent subcloning or sequencing contaminating bacterial chromosomal DNA and yields the circular plasmid, COSMID and BAC forms that result in maximum transformation efficiencies.

Reader Enquiry No. 112

Novagen's new BacVector **protein expression** system offers several time-saving features for baculovirus expression. The BacVector-1000 and -2000 will triple-cut virus DNA for recombinant frequencies near a stated 100 per cent. Ready-to-use Sf9 frozen insect cells that can be thawed and used immediately for cell transfection, plaque assays or cell culture. A variety of pBAC plasmids, with or without a *gus* reporter gene for positive identification of recombinants, and optional fusion tags for secretion and fast, economical protein purification. The complete system includes the transfection kits, ligation-independent cloning kits and a baculovirus display system for expression of complex proteins on the virus surface.

Reader Enquiry No. 113

The Oncor Trap-eze **telomerase detection kit** is a highly sensitive *in vitro* assay for detecting telomerase activity in cells or tissues. This PCR-based telomerase detec-

tion method has improved in its ability to modify primer sequence includes an internal control for each test sample being analyzed, thus eliminating the need for 'hot start' PCR and enhancing data interpretation. Other features include an optimized protocol that allows radioactive and non-radioactive detection. The company suggests that the product should find use in cancer and aging research.

Reader Enquiry No. 114

Hybridization techniques

Vysis has introduced HYBrite, a new open system for hands-free denaturation and hybridization of *in situ* DNA probe procedures. The system is designed to eliminate several steps and to reduce the hands-on time required during conventional fluorescence *in situ* hybridization procedures. It has a capacity to store up to ten user-defined programs, as well as a user guide that provides step-by-step instructions and recommended parameters for the company's DNA probes. The system has a 12-slide capacity and heats



Vysis' HYBrite for *in situ* hybridization.

slides through two temperature ranges with precise control of heating and cooling. A user-defined program is designed to support a variety of specimen types such as cultured lymphocytes, bone marrow and paraffin-embedded tissue.

Reader Enquiry No. 115

A new range of **hybridization ovens**, developed by Heraeus Instruments allows identical or almost identical nucleotide sequences to be identified. Herahybrid 6 is the standard oven that accepts six hybridization bottles and has a fixed speed of seven revolutions per minute. Herahybrid 12 is the larger version, which doubles this capacity and offers variable speed selection from 0 to 15 r.p.m. To optimize the hybridization process and to help ensure reproducible results, both ovens offer full microprocessor control. Temperatures are continuously adjustable up to 99.9 °C with built-in over-temperature protection enabling the ovens to safely operate while unattended. For safe loading and unloading of bottle racks a door switch automatically stops the turn-

ing movement of the bottle rack as soon as the door is opened. A switch can also be operated to select the desired removal position for hybridization bottles. Both ovens feature corrosion-resistant stainless steel interiors with a removable drip tray and bottle rack for easy access and cleaning. Hybridization bottles in 100-mm, 200-mm and 300-mm sizes, as well as a stand that holds six bottles, are available as accessories.

Reader Enquiry No. 116

Boehringer Mannheim now offers a free, updated version of its **non-radioactive *in situ* hybridization application (ISH) manual**. The 214-page, full-colour manual describes step-by-step experimental protocols for non-radioactive alternatives for ISH on isolated chromosomes, cells, tissue, sections and whole mounts, as well as



Boehringer Mannheim: manual dexterity.

others. New products described in the manual discuss how labelling and detection of non-radioactive probes can be employed, these products include nick-translation labelling of the DNA probe and the DIG-, biotin, and fluorescein High Prime reaction mixes for random-primed labelling of DNA probes.

Reader Enquiry No. 117

cDNA synthesis

R&D Systems offers a new **MLV reverse transcriptase enzyme** with low RNase H activity that can be used to prepare first strand cDNA in high yields with

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READER ENQUIRY NO. 69

long cDNA products. The complete kit includes all of the reagents required for cDNA synthesis; these are tested to ensure the absence of harmful RNases that could degrade the template. Moreover, there is a choice of priming strategies with control reagents that are designed to assist with synthesis and amplification. The kit contains random and oligo-d(T) primers and, as an alternative, target-specific primers. This system allows the cDNA synthesis to be conducted directly from a PCR template without purification.

Reader Enquiry No. 118

Clontech's new PCR cDNA library construction kit, called CapFinder, is designed to allow researchers to construct representative cDNA libraries highly enriched for full-length clones from only 50 ng of total RNA. This technology uses long-distance PCR and the CapSwitch oligonucleotide to generate high yields of full-length, double-stranded cDNA from which libraries can be constructed. According to the manufacturer, libraries can be created from very small samples, such as flow-sorted cells, tissue sections, embryonic cell samples and biopsies.

Reader Enquiry No. 119

Stratagene's cDNA synthesis kits have been designed for fast, efficient construc-

tion of directional cDNA libraries. The kits use the company's Lambda Zap vectors, enabling researchers to use in vivo excision to convert lambda clones or libraries into single- or double-stranded libraries. The use of Pfu DNA polymerase for end polishing, instead of Klenow DNA polymerase, results in higher quality, more efficiently prepared cDNA libraries, according to the manufacturer. This polymerase does not exhibit extendase activity, so it does not create unwanted 3' overhangs. When used for end polishing, Pfu DNA polymerase creates higher numbers of blunt-ended molecules available for adapter ligation, significantly improving the efficiency of cloning blunt-ended fragments. The high thermostability of the polymerase makes it possible to perform the end-polishing reaction immediately after second-strand synthesis, reducing the time needed to construct a library. There are no time-consuming ethanol precipitations needed at this step, preventing the loss of DNA samples. Stratagene's line of cDNA synthesis kits include the Zap-cDNA and ZapExpress cDNA synthesis kits and the HybriZap two-hybrid cDNA Gigapack cloning kit.

Reader Enquiry No. 120

Miscellaneous

The new FL3000 Spectrometer from Thermo Separation Products has been designed with improvements aimed at greater sensitivity. That is the major determinant in fluorescence detector performance. The illuminated volume of the FL3000's flow cell has been increased from 3 pl to 8 pl, providing a more intense signal from a given concentration of fluorescent material. Because most fluorescence applications require an excitation wavelength between 300 and 400 nm, TSP has optimized the grating blaze and PMT spectral response for improved sensitivity in this range. Serial control and data collection by TSP's PC1000 software should allow the programming of a series of automated HPLC runs from a single keyboard, performing the sequence and storing the data in the computer. There is post-run data retrieval and analysis, boosting productivity by simplifying access to and management of the data. The FL3000 displays post-run excitation and emission wavelengths and fluorescence intensity on a front panel LCD after a stand-alone run. An operator can manually browse through a stored scan and view the change in fluorescence level as the wavelength is changed. Variable excitation and emission slits give the operator the flexibility to customize the instrument's performance. With time-programmable excitation and emission ratings, applications can be tailored to detect compounds at their excitation and emission maxima.

Reader Enquiry No. 121

Dako's new liquid-stable amplification uses an enzyme cycling system that is designed to amplify the signal generated by alkaline phosphatase. According to the manufacturer, amplification factors of 100-fold over the primary enzyme signal can be achieved. The system is suitable for all immunoassay formats where a coloured product is required, such as tubes, beads and microplates. AmpliQ is designed to be user-friendly with ready-to-use liquid reagents in dropper bottles and a procedure that requires only one timed step.

Reader Enquiry No. 122

Easyject Basic is offered as an inexpensive electroporation system transforming *Escherichia coli* and other gram-negative bacteria. The unit is designed for simplicity, the user inserts the cuvette and operates the start button to initiate the electroporation. The voltage and pulse parameters are pre-set and optimized to provide a high transformation efficiency. The device incorporates the power supply and electronic control in one small unit providing a safe, simple system, which maintains a small footprint. It can be used directly from the main power supply or from the built-in rechargeable battery thereby, giving added flexibility for field, portable or sterile applications.

Reader Enquiry No. 123

CPG also offers Label-It 3' biotin end-labelling kit, which is said to provide a simple, one-hour protocol for efficient labelling of any oligonucleotide, including PCR and sequence primers, as well as other DNA molecules with biotin-14-dCTP. This kit includes all of the reagents required to perform 25 end-labelling reactions. In addition to the reagents needed to perform the labelling reactions, the kit contains a non-isotopic assay for assessing the efficiency of the biotin incorporation on the nucleic acid. The labelling reactions are carried out using terminal deoxynucleotidyl transferase to produce a labelled probe for use in non-isotopic hybridization assays. One to four biotinylated nucleotides are added, with the majority of oligo being converted to a labelled nucleic acid with mostly two and three additions. Greater than 80 per cent of DNA can also be used in solid phase-based nucleic acid applications using the biotin as an anchor for binding to streptavidin-solid supports. This kit eliminates the need for purchasing costly biotin-modified oligonucleotides.

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These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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